Identification and Solution Structures of a Single Domain Biotin/Lipoyl Attachment Protein from *Bacillus subtilis*

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Protein biotinylation and lipoylation are post-translational modifications, in which biotin or lipoic acid is covalently attached to specific proteins containing biotin/lipoyl attachment domains. All the currently reported natural proteins containing biotin/lipoyl attachment domains are multidomain proteins and can only be modified by either biotin or lipoic acid *in vivo*. We have identified a single domain protein with 73 amino acid residues from *Bacillus subtilis* strain 168, and it can be both biotinylated and lipoylated in *Escherichia coli*. The protein is therefore named as biotin/lipoyl attachment protein (BLAP). This is the first report that a natural single domain protein exists as both a biotin and lipoic acid receptor. The solution structure of apo-BLAP showed that it adopts a typical fold of biotin/lipoyl attachment domain. The structure of biotinylated BLAP revealed that the biotin moiety is covalently attached to the side chain of Lys35, and the bicyclic ring of biotin is folded back and immobilized on the protein surface. The biotin moiety immobilization is mainly due to an interaction between the biotin ureido ring and the indole ring of Trp12. NMR study also indicated that the lipoyl group of the lipoylated BLAP is also immobilized on the protein surface in a similar fashion as the biotin moiety in the biotinylated protein.

The biotin/lipoyl attachment domain (IPR000089) is a signature structural motif, which has a conserved lysine residue that can bind biotin or lipoic acid. This domain can be found in enzymes that require biotin or lipoic acid as cofactor (1). Biotin plays a catalytic role in carboxyl transfer reactions. It is covalently attached to a lysine residue, via an ε-amino group, in enzymes such as pyruvate carboxylase, acetyl-CoA carboxylase, and propionyl-CoA carboxylase, etc. (2). This process, called biotinylation, is catalyzed by biotin-protein ligase (BPL)² (1, 3).

Biotin/Lipoyl Attachment Protein (BLAP)

Lipoic acid can serve as cofactor in a variety of proteins, such as 2E acyltransferases (E2p) in the pyruvate dehydrogenase complex and H-protein of the glycine cleavage system (4, 5). It is also covalently bound via an amide linkage to a lysine group. This process, named lipoylation, is catalyzed by lipoate-protein ligase (LPL) (1, 6). It is known that BPLs have broad substrate ranges. Mammalian biotinyl domain can be biotinylated by bacterial BPL and vice versa. The situation for LPLs is also similar (1).

The structures of several biotin/lipoyl attachment domains have been determined. Two of them are biotinyl domains, the C-terminal domain of *Escherichia coli* biotin carboxyl carrier protein (BCCP) and the biotinyl domain of 1.3 S subunit of transcarboxylase from *Propionibacterium shermanii* (7–10). Several structures of lipoyl domains have also been reported (11–15). All biotinyl domains and lipoyl domains share a very similar overall fold, which is a flattened β-barrel formed by two β-sheets. The conserved lysine residue is located at the tip of a tight β-turn.

Although biotinyl and lipoyl domains are quite similar in their three-dimensional structures, there is no natural protein currently reported to be recognized by both BPL and LPL. However, it is found that the biotinyl domain of *E. coli* BCCP can be lipoylated after its protruding thumb is removed, and the removal does not affect its biotinylation property (16).

All the proteins containing a biotin/lipoyl attachment domain, reported so far, are multidomain proteins. The other domains are required for them to be functional (10, 17–19). Here we report the identification and the structural characterization of a single domain protein (GenBank™ accession number NP_570905) with 73 amino acid residues from *Bacillus subtilis* strain 168. Because the size of this protein only corresponds to the sizes of the biotin/lipoyl attachment domains and can be both biotinylated and lipoylated in *E. coli*, we named it as biotin/lipoyl attachment protein (BLAP).

MATERIALS AND METHODS

Cloning, Expression, and Purification of BLAP—The gene of BLAP was amplified from *B. subtilis* strain 168 genome by PCR and cloned into protein expression vector pET-21a(+). The recombinant plasmid was transformed into *E. coli* BL21(DE3)/pLysS for protein expression. A single colony of the bacteria was cultured overnight in 2 ml of LB medium at 35 °C, with 100 mg/liter ampicillin and 34 mg/liter chloramphenicol. The cells were harvested by centrifugation and treated with lysozyme. BLAP and lipoylated BLAP (LPL-BLAP, WT), wild-type; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.

The atomic coordinates and structure factors (code 1Z7T, 1Z6H, 2B8F, 2B8G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
were collected by centrifugation and transferred into 250 ml of M9 minimal medium to continue incubation. When the cell density reached A₆₀₀ of 1.0, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM to induce protein expression. d-Biotin was added into M9 medium to express the biotinylated protein, and α-lipoic acid was added for the lipoylated protein production. ¹⁵NNH₄Cl and [¹³C]glucose were used in M9 medium for preparing ¹⁵N/¹³C-labeled protein samples. BLAP was purified with a Q-Sepharose anion exchange column followed by gel filtration using ACTA fast protein liquid chromatography (Amersham Biosciences).

Reverse Transcription-PCR—The total mRNA was extracted from cells of B. subtilis strain 168 grown in LB media with 600 mg/liter streptomycin and harvested at A₆₀₀ of 0.6. Reverse transcription was carried out with 2.5 μg of total RNA using superscript II reverse transcriptase and random hexamers as primers. Real time PCR was performed using the Qiagen SYBR Green™ PCR master mix kit according to the manufacturer’s instruction. Each reaction was repeated three times.

Western Blot—The primary antibody was from the rabbit serum after 45 days of immunization by four times intramuscular injection of purified recombinant BLAP protein. Anti-Rb IgG (H + L) horseradish peroxidase was used as secondary antibody. Western blot analysis was performed according to the standard method (20) with purified BLAP as positive control.

NMR Spectroscopy—All the NMR samples contained about 1.2 mM protein in 50 mM phosphate buffer, pH 7.0, with 1 mM EDTA and 0.01% NaN₃, in 90:10% H₂O/D₂O. 2,2-Dimethyl-2-silapentanesulfonic acid was added as the chemical shift reference. All the NMR spectra were collected at 298 K on a Bruker AVANCE 600 MHz NMR spectrometer. The backbone chemical shift assignments were obtained based on two-dimensional ¹H-¹⁵N HSQC, three-dimensional HNCACB, three-dimensional CBCA(CO)NH, three-dimensional HNCA, and three-dimensional HNCO experiment data, whereas the side chain resonance assignments were obtained from two-dimensional ¹H-¹³C HSQC, three-dimensional HCH-COSY, three-dimensional HBHA(CO)NH, three-dimensional ¹³C total correlation spectroscopy-HSQC, three-dimensional HCC(CO)NH, and three-dimensional CC(CO)NH experiment data (21). Three-dimensional ¹³C-NOESY-HSQC and three-dimensional ¹⁵N-NOESY-HSQC spectra (22) were collected with mixing times of 60 and 120 ms, respectively. The side chain assignments of aromatic residues were obtained based on three-dimensional ¹³C-NOESY-HSQC experiment data for the aromatic carbons. Two-dimensional ¹³C/¹⁵N-filtered NOESY spectra (23, 24) (mixing time of 200 ms) were collected for the assignments of proton resonances from the attached biotin or lipoate moiety. One-dimensional ¹H NMR spectrum, two-dimensional NOESY, and double quantum-filtered correlation spectroscopy data were used for obtaining the proton chemical shifts of free lipoic acid. The steady state heteronuclear [¹H]-¹⁵N NOE experiments (25) were performed in the presence and absence of a 3-s proton pre-saturation period (26, 27). All NMR spectra were processed using NMRPipe (28) and analyzed using NMRView (29).

| TABLE 1 |
| --- |
| **Restraints and structural statistics for apo- and bli-BLAP** |
| **Apo-BLAP** | **bli-BLAP** |
| Distance restraints | 1093 | 1191 |
| Intra-residue (i – i) = 0 | 678 | 721 |
| Sequential (i – i – 1) | 260 | 313 |
| Medium (2 ≤ i – i ≤ 4) | 1003 | 1129 |
| Long range (i – i ≥ 5) | 697 | 806 |
| Total | 3731 | 4160 |
| Dihedral angle restraints | | |
| φ | 34 | 35 |
| ψ | 34 | 37 |
| χ¹ | 41 | 45 |
| Total | 109 | 117 |
| Hydrogen bond | 28 | 30 |
| Structure statistics (20 structures) | | |
| Violation | | |
| NOE violation (≥ 0.2 Å) | 0 | 0 |
| Maximum NOE violation (Å) | 0.14 | 0 |
| Torsion angle violation (≥ 5°) | 0 | 0 |
| Energy | | |
| Mean restraint violation energy (kcal mol⁻¹) | 2.98 | 5.35 |
| Mean AMBER energy (kcal mol⁻¹) | -3746.74 | -3890.90 |
| PROCHECK statistics | | |
| Residues in core regions (%) | 86.7 | 83.9 |
| Residues in allowed regions (%) | 13.3 | 15.6 |
| Residues in generously allowed regions (%) | 0 | 0.2 |
| Residues in disallowed regions (%) | 0 | 0 |
| r.m.s.d. from mean structure (residues 2–69) | | |
| Backbone heavy atoms (Å) | 0.32 ± 0.07 | 0.30 ± 0.10 |
| All heavy atoms (Å) | 0.91 ± 0.13 | 0.89 ± 0.14 |

Structure Calculations—The solution structures were calculated using CYANA (30) and refined with AMBER (31). The distance restraints were obtained from analysis of NOESY data using SANE (32). Dihedral angle restraints (φ and ψ) were determined based on analysis results from TALOS (33) and CSI (34). χ¹ angles and stereo-specific assignments of H² were determined based on NOE intensity information from three-dimensional ¹⁵N-NOESY-HSQC and three-dimensional ¹³C-NOESY-HSQC data. Hydrogen bond constraints were used based on NOE analysis and the secondary structure predictions by CSI.

A total of 200 structures was calculated with CYANA, and 100 of them with the lowest target functions were selected for further refinement with AMBER. The final 20 structures with the lowest AMBER energies were selected for structure representation. The final structures were analyzed using MOLMOL (35) and PROCHECK-NMR (36). The mean square deviation (r.m.s.d.) data were calculated using SUPPOSE (37). The mean structures were generated using SUPPOSE and energy-minimized with AMBER.

RESULTS

Cloning and Recombinant Expression of BLAP in E. coli—The DNA fragment encoding BLAP was successfully amplified from the genome DNA of B. subtilis strain 168 and was ligated into pET expression vector. DNA sequencing results showed that the coding sequence of the cloned BLAP gene is identical to the published sequence in the NCBI data base (GenBank™ accession number NC_000964).

BLAP was expressed in E. coli with a typical protein yield of 60 – 80 mg/liter. Mass spectrometry (MS) analysis showed that
the molecular mass of the purified protein is 7913 Da, which is 128 Da less than the theoretical value. This mass difference is because of the missing of the first methionine, which was confirmed by N-terminal protein sequencing analysis. The MS data indicated that the expressed BLAP is without any post-translational modification.

When the bacteria were grown in M9 minimal medium supplied with 40 mg/liter biotin, both apo and biotinylated (btl) BLAP were obtained. Apo-BLAP and btl-BLAP can be separated on an anion exchange column because btl-BLAP has one less positive charge than apo-BLAP. At pH 9.0, btl-BLAP was eluted from the Q-Sepharose column at 130 mM NaCl. Interestingly, lipoylated (lpl) BLAP was produced when the cells were cultured in M9 minimal medium with 40 mg/liter lipoic acid. The molecular masses of both btl-BLAP and lpl-BLAP were verified by MS. The molecular weight difference between btl-BLAP and apo-BLAP is 225.9 Da and that between lpl-BLAP and apo-BLAP is 188.5 Da. These values agreed with the corresponding theoretical values.

Verification of BLAP Expression in B. subtilis—To determine whether BLAP is expressed in B. subtilis, we first tried to detect the mRNA of BLAP in B. subtilis total mRNA extraction. After reverse transcription, real time PCR was carried out, and the results showed that the mRNA of BLAP has a transcription level close to that of B. subtilis 16 S rRNA gene rrnO (data not shown). The fluorescence threshold was set at 0.02, and the threshold cycle (CT) for BLAP coding sequence and rrnO is 28.8 and 28.2, respectively. Melting curve analysis indicated that the PCR product is a single fraction, which means no nonspecific PCR occurred. Western blot was used to verify the protein expression of BLAP in B. subtilis. Cells of B. subtilis strain 168 were cultured in 500 ml of LB media until the $A_{600}$ reached 1.5. The bacteria were harvested, and the cells were broken by using a French press. The supernatant was then loaded onto a Q-Sepharose column at pH 9.0, and the fraction eluted with 0.2 M NaCl was collected and concentrated to 0.5 ml. 7.5 ml of this concentrated fraction was used for Western blot analysis. A single band was detected from this sample by immunoblot using monoclonal antibody generated against the recombinant BLAP. This single band appeared at exactly the same position as the purified recombinant BLAP.

Solution Structure of Apo-BLAP—Nearly all the chemical shift assignments were obtained for apo-BLAP except for Asn70, whose NH signal was missing in the two-dimensional $^1$H-$^1$N HSQC spectrum. The solution structure of apo-BLAP was calculated based on NOE, dihedral angle, and hydrogen bond restraints (Table 1). The superimposition of the final 20 structures with the lowest AMBER energies for apo-BLAP, together with a ribbon diagram of the energy minimized mean structure, are shown in Fig. 1. Residues 2–69 of apo-BLAP form a well defined structure, whereas the last four residues are less defined. The amount of btl-BLAP or lpl-BLAP produced in E. coli is dependent on the incubation time after isopropyl $\beta$-D-galactopyranoside induction. When the bacteria were incubated for 6.5 h after induction, about 10% BLAP was biotinylated (~50% lipooylated). When it was elongated to 16 h, about 50% BLAP was biotinylated (~75% lipooylated). The rates of biotinylation and lipoylation for BLAP in E. coli are comparable with the biotinylation rate of the biotinyl domain of E. coli BCCP and the lipoylation rate of the lipoyl domain of E. coli E2p, respectively (38).
residues are flexible. Structural statistics indicated the solution structure is in very good quality (Table 1).

Apo-BLAP adopts a typical biotin/lipoyl attachment domain fold, which is a flat barrel formed by two β-sheets (Fig. 1). Residues 3–4 (β1), 21–22 (β3), 46–47 (β6), 48–51 (β7), and 64–68 (β9) form one anti-parallel β-sheet (S1), whereas residues 9–14 (β2), 27–33 (β4), 36–41 (β5), and 58–59 (β8) compose another anti-parallel β-sheet (S2). Strands β4 and β5 are connected by a type III′ β-turn, and the conserved active site MKM residues are located at this β-turn in S2. Lys35 at the tip of this β-turn is fully exposed with its flexible side chain sticking out.

Solution Structure of btl-BLAP—We obtained nearly complete chemical shift assignments for btl-BLAP except Val3 and the NH of Asn70. An extra signal appeared in the two-dimensional 1H-15N HSQC spectrum of btl-BLAP, which was assigned to the e-NH of the biotin-attached Lys35.

Comparing the two-dimensional 1H-15N HSQC spectrum of btl-BLAP with that of apo-BLAP, it is found that the backbone NH signal of Lys35 has the largest chemical shift change (Fig. 2). Meanwhile, NH signals from residues near Lys35 (Ile30–Ile38) have significant chemical shift changes because of the biotinylation. Chemical shift changes are also observed for NH signals of residues 7, 9–15, 28, 56, and 60, as well as the NH2 signals of Asn10 (Fig. 2).

The 1H chemical shifts of the attached biotin were all assigned and are listed in Table 2 and Structure 1. The attached biotin moiety showed significant 1H chemical shift changes as compared with free biotin. Most of the 1H chemical shifts of the attached biotin are decreased, and generally, those further to the carboxyl group have larger decrease in chemical shift. The chemical shifts for HN3 and one proton of H10 are increased.

The superimposition of the 20 btl-BLAP structures with the lowest AMBER energies is shown in Fig. 3A. The structural statistics for btl-BLAP are listed in Table 1. The structures of apo-BLAP and btl-BLAP are very similar (Fig. 3B). The r.m.s.d. (residues 2–69) for backbone heavy atoms between the mean structures of the two forms is 0.83 Å. The major structural displacement occurs at the end of β4, the biotin-attached turn, and the first half of β5. The β-turn with the attached biotin is converted into a type I′ β-turn from a type III′ β-turn in apo-BLAP. As a result, the end of β4 moves closer to the beginning of β2. The biotinylation of BLAP stabilizes the side chain of Lys35, which leads to the side chain heavy atom r.m.s.d. of Lys35 (not including biotin moiety) for the ensemble of structures reduced from 1.72 Å in apo-BLAP to 0.69 Å in btl-BLAP.

Conformation of the Attached Biotin—The biotin moiety is well defined in the btl-BLAP structure. The heavy atom r.m.s.d. from mean for the biotin group is 0.61 Å in the ensemble of structures. The bicyclic ring of the attached biotin is folded back and immobilized on the protein surface, mainly on top of β4. The side chains of Glu32 (on β4) and Glu37 (on β5) are pushed away from the biotin for its bicyclic ring to fit (Fig. 4, A and B). The NH2 signals of Asn10 in the two-dimensional structures are from mean for the biotin group and are listed in Table 1.
$^{1}$H-$^{15}$N HSQC spectrum of btl-BLAP show a very large shift compared with those of apo-BLAP. In the btl-BLAP structure, the side chain of Asn$^{10}$ moves toward the side chain of Glu$^{32}$, and the NH$_2$ group of Asn$^{10}$ is in a position to form a hydrogen bond with the carboxyl group of Glu$^{32}$ in all 20 structures of btl-BLAP. Most interestingly, the indole ring of Trp$^{12}$ turns almost 90° toward the biotin to face its ureido ring. The conformation changes for the side chains of Asn$^{10}$ and Trp$^{12}$ on $\beta_2$ are probably responsible for the NH chemical shift changes for residues 9–15. The small chemical shift changes observed for NH signals of Val$^{28}$, Gly$^{56}$, and Asn$^{60}$ could be attributed to a secondary effect from the conformation changes of Asn$^{10}$ and Trp$^{12}$ side chains, because of the hydrogen bond network of the S2 $\beta$-sheet.

The binding site of the biotin bicyclic ring is mainly negatively charged (Fig. 3C), and no specific interaction (hydrogen bond, hydrophobic interaction, etc.) between the biotin bicyclic ring and the protein can be identified. Analysis of NOEs between the biotin and the protein showed that all the protons on the biotin bicyclic ring have NOEs with the indole ring of Trp$^{12}$. The distance between the center of the Trp$^{12}$ hexagon ring and the center of the biotin ureido ring is about 3.8 Å, and the angle between the planes of the two rings is 29.5° in the mean structure.

The protons on the biotin bicyclic ring and those close to this ring, whose chemical shifts decrease significantly, must be within the shielding cone of the indole ring of Trp$^{12}$. Their chemical shift decreases should be a result of aromatic ring current effect, although the HN3 of biotin is probably located outside of the shielding cone and therefore has an increased chemical shift. The distances between H10 protons and the centers of either the pentagon or the hexagon rings of the indole group are over 10 Å. Therefore, the chemical shift changes for H10 are probably related to local conformation change because of the immobilization of biotin.

The steady state heteronuclear $^1$H-$^{15}$N NOE value of the $\epsilon$-N$^\delta$ of Trp$^{12}$ changes from 0.50 ± 0.02 in apo-BLAP to 0.71 ± 0.02 in btl-BLAP. This indicates that the motion of the indole ring is somewhat restricted upon the attachment of biotin. As only this indole ring moved toward biotin and all other nearby side chains moved away, we suspected that the immobilization of the biotin is mainly due to an interaction between the
dimensional $^1$H-$^{15}$N HSQC spectra of W12M apo- and btl-BLAP. For W12M BLAP, biotinylation only causes chemical shift changes for the backbone NH signals of residues at the biotin-attaching site, residues 34–37. Unlike the situation in WT BLAP, the backbone NH chemical shifts for residues 9–15 in W12M BLAP almost have no change upon biotinylation. The NH$_2$ signals of Asn$^{10}$ are shifted upon biotinylation, but the scales of change are much smaller than those in WT BLAP. In addition, the steady state heteronuclear $^1$H-$^{15}$N NOE value of the $\epsilon$-$N^{14}$ of Lys$^{35}$ is $-0.54 \pm 0.02$ for W12M btl-BLAP, whereas it is $0.54 \pm 0.02$ for WT btl-BLAP. These values imply that the biotin moiety and the side chain of Lys$^{35}$ are flexible in W12M btl-BLAP, and it is a result of a single residue mutation, W12M. Indeed, the interaction between the indole ring of Trp$^{12}$ and the
biotin ureido ring is responsible for the immobilization of the biotin moiety in WT BLAP. The nature of this interaction is probably electrostatic interaction.

**NMR Study of lpl-BLAP**—Backbone resonance assignments were carried out for lpl-BLAP. Except that Asn70 lacked an NH signal, all the possible backbone NH and side chain NH$_2$ resonances in the two-dimensional $^1$H-$^1$H HSQC spectrum were assigned. The e-NH signal of the lipoate attached Lys35 was also identified (Fig. 6).

Similar to btl-BLAP, the residues with obvious NH chemical shift changes because of lipoylation mainly exist in two regions as follows: one is near Lys35 (Ile30, Glu32–Ile38) and the other is near Trp12 (Asn10–Lys13). Val14, Gly56, and Asn60 also show small chemical shift changes. The residues with obvious chemical shift variations because of biotinylation or lipoylation are almost identical. Actually, the NH chemical shift differences between lpl-BLAP and btl-BLAP are much smaller than those between them and the apo-BLAP for residues with very large NH chemical shift changes (residues 10, 35 and 36) (Fig. 6).

The $^1$H chemical shifts of the attached free lipoic acid were also assigned (Table 3 and Structure 2). All the $^1$H chemical shifts are decreased for the attached lipoic acid compared with those of the free lipoic acid except for H9. Those further from the carboxyl group have larger chemical shift changes. This is similar to the case of the attached biotin in btl-BLAP. The significant chemical shift decreases are probably also because of the ring current effect of Trp12.

The 1H chemical shifts of the attached and free lipoic acid were also assigned (Table 3 and Structure 2). All the 1H chemical shifts are decreased for the attached lipoic acid compared with those of the free lipoic acid except for H9. Those further from the carboxyl group have larger chemical shift changes. This is similar to the case of the attached biotin in btl-BLAP. The significant chemical shift decreases are probably also because of the ring current effect of Trp12.

The steady state heteronuclear [$^1$H]-$^1$N NOE value of the e-NH of Trp12 increases from 0.50 ± 0.02 in apo-BLAP to 0.61 ± 0.02 in lpl-BLAP. Therefore, we believe that the attached lipoate moiety is also immobilized on the protein surface, and it is in a similar conformation as the attached biotin in btl-BLAP. We suspected that the immobilization of lipoate moiety is also mainly due to an electrostatic interaction between the rings of lipoic acid and Trp12.

**DISCUSSION**

The Existence of BLAP—The *B. subtilis* BLAP (GenBank™ accession number NP_570905) was initially annotated as a BCCP. Unlike other bacterial BCCPs that normally have 150–

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**TABLE 3**

| Resonance$^a$ | Chemical shift (ppm) |
|--------------|----------------------|
|              | **Free lipoic acid**$^b$ | **Bound lipoic acid** |
| H3           | 3.18, 3.25            | 2.09, 2.43            |
| H4           | 2.00, 2.49            | 1.14, 1.72            |
| H5           | 3.72                  | 3.33                  |
| H6           | 1.76, 1.64            | 1.20, 1.17            |
| H7           | 1.42, 1.42            | 1.17, 1.04            |
| H8           | 1.58, 1.58            | 1.55, 1.45            |
| H9           | 2.18, 2.18            | 2.19, 2.17            |

$^a$ The IUPAC nomenclature for the biotin is given in Structure 2.

$^b$ Chemical shifts for free lipoic acid was determined in 50 mM phosphate buffer, pH 7, at 298 K.

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**FIGURE 6.** Overlay of the two-dimensional $^1$H-$^1$N HSQC spectra of apo-BLAP (blue), btl-BLAP (red), and lpl-BLAP (green). Residues with obvious chemical shift changes are labeled by the one-letter amino acid code and residue number.

**FIGURE 7.** Alignment of protein sequences with high homology and similar length to BLAP.
160 amino acid residues, BLAP is only 73 residues in length. So its size is about the same as those of biotinyl domains of bacterial BCCPs. The sequence identities between BLAP and the biotinyl domains of BCCPs of *B. subtilis* and *E. coli* are 23.9 and 26.1%, respectively.

The entry for GenBank™ accession number NP_570905 was later discontinued in the NCBI protein data base after we have cloned the gene of BLAP. In this study, we have proved the existence of BLAP in *B. subtilis* at both the mRNA level and the protein level.

About 30 homologous protein sequences were found when we searched the nonredundant protein data base with the *B. subtilis* BLAP sequence on Biology Workbench. These protein sequences all have over 60% positive matches with the *B. subtilis* BLAP, and their sequence lengths are all less than 90 amino acid residues (Fig. 7). The MKM motif and the glutamic acid at the −3 position with respect to the target lysine, which is very important for biotinylation, are highly conserved (Fig. 7). All of them are from prokaryotes, except one is from *Danio rerio* (GenBank™ accession number XP_701405). Therefore, BLAP is not only restricted to *B. subtilis* but also exists in other species.

**FIGURE 8.** A, structure comparison of biotinyl domains and lipoyl domains in the apo form. B. BLAP is for BLAP from *B. subtilis* strain 168; *E. BCCP* is the biotinyl domain of the *E. coli* BCCP (Protein Data Bank code 1A6X); *P. 1.3 S* is the biotinyl domain of the 1.3 S subunit of transcarboxylase from *P. shermanii* (Protein Data Bank code 1DCZ); A. OGDH is the lipoyl domain of the 2-oxoglutarate dehydrogenase complex from *A. vinelandii* (Protein Data Bank code 1GHJ); H. BCDH is the lipoyl domain of the E2 component of human mitochondrial branched chain α-keto acid dehydrogenase (Protein Data Bank code 1K8M); *E. PDH* is the innermost lipoyl domain of the pyruvate dehydrogenase from *E. coli* (Protein Data Bank code 1QJO). Loop 1 is indicated in red. Loop 2 (protruding thumb) is indicated in magenta. The target lysine is displayed in blue. B, protein sequence comparison of BLAP with the biotinyl domains and lipoyl domains mentioned above. The corresponding secondary structure elements are indicated.

The bicyclic ring of biotin is also attached on the protein surface near the protruding thumb of the biotinyl domain of *E. coli* BCCP (7). It was first proposed that a hydrogen bond between the HN₂ of biotin and the OHex of Thr⁹₂⁴ was the reason for the immobilization of biotin based on the crystal structure (7), although NMR study results of the biotinyl domain of *E. coli* BCCP did not support this hydrogen bond in solution structure (9). However, the immobilization of the attached biotin in *E. coli* BCCP was generally attributed to the interaction between biotin and the protruding thumb (8–10).

By Examining the structure of the biotinylated biotinyl domain of *E. coli* BCCP, we found that Tyr⁹² of *E. coli* BCCP is in a similar position to Trp¹² of btl-BLAP. The phenyl ring of Tyr⁹² in *E. coli* BCCP is also facing the ureido ring of biotin, and the distance between the centers of the two rings is 3.7 Å (Fig. 4C). In addition, it is also reported that extensive NOEs have been
observed between the phenyl ring of Tyr\(^{92}\) and every proton of the biotin bicyclic ring (9). Based on this information, we propose that the immobilization of the biotin moiety in *E. coli* BCCP is also because of an interaction between the phenyl ring of Tyr\(^{92}\) and the biotin ureido ring, similar to the case for btl-BLAP described above.

On the other hand, it has been reported that the attached biotin is flexible in the biotinyl domain of the 1.3 S subunit of transcarboxylase from *P. shermanii* (39). The structure of the biotinyl domain of the 1.3 S subunit shows that there is no aromatic residue in similar positions as Trp\(^{12}\) of BLAP or Tyr\(^{92}\) of *E. coli* BCCP (Fig. 4D). This should explain the flexibility of the attached biotin in the biotinyl domain of the 1.3 S subunit.

**Substrate Recognition by BPL and LPL**—Although biotinyl domains and lipoyl domains share a very similar overall fold, it is a general belief that BPL and LPL enzymes can distinguish biotinyl and lipoyl domains as substrates (1). Several studies have been carried out to reveal the mechanisms for the enzyme specificities of BPL and LPL (16, 38, 40, 41).

Mutagenesis study has shown that removing the protruding thumb, which lipoyl domains do not have, from the biotinyl domain of *E. coli* BCCP made it an efficient substrate for LPL, and it still retained the ability to be recognized by BPL (16). So the protruding thumb of *E. coli* BCCP prevents it from being lipoylated. Also, based on sequence and structure comparisons, it has been proposed that loop 1 between strands 1 and 2 is responsible for BPL not recognizing lipoyl domains (42). This loop in lipoyl domains is generally a few residues longer than that in biotinyl domains.

BLAP can be both biotinylated and lipoylated, which indicates that it has the structural characteristics recognized by both BPL and LPL. In Fig. 8, the structure of BLAP is compared with two biotinyl domain structures and three lipoyl domain structures. The fact that BLAP lacks the protruding thumb and has shorter loop 1 makes it a good substrate for both BPL and LPL.

The protruding thumb of the biotinyl domain of *E. coli* BCCP is not a common feature for all biotinyl domains. It only exists in the biotinyl domains of bacterial acetyl-CoA carboxylase (1). Other biotinyl domains, such as that of the 1.3 S subunit from *P. shermanii*, have much shorter loops at the corresponding places, as does BLAP. It is possible that this kind of biotinyl domain can also be an effective substrate for LPL.

**Biological Implications**—Being both biotinylated and lipoylated efficiently does not mean that BLAP is involved in carboxylase or dehydrogenase complex, as the biotin/lipoate attachment protein (24). Iwahara, J., Wojciak, J. M., and Clubb, R. T. (2001) *Annu. Rev. Biochem.* 69, 961–1004

In summary, we have identified a new protein (BLAP) from *B. subtilis* that can be both biotinylated and lipoylated. Unlike all other biotin/lipoyl attachment domain-containing proteins, it is a single domain protein. However, its biological function is still not clear, and further studies are needed to reveal the biological role of BLAP.

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