Protein Science (1999), *8*:921–929. Cambridge University Press. Printed in the USA.

A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation

DOROTHY BECKETT,[1] ELENA KOVALEVA,[1] AND PETER J. SCHATZ[2]

[1]Department of Chemistry and Biochemistry, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, Maryland 21250
[2]Affymax Research Institute, 4001 Miranda Ave., Palo Alto, California 94304

(RECEIVED October 26, 1998; ACCEPTED January 8, 1999)

Abstract

The *Escherichia coli* biotin holoenzyme synthetase, BirA, catalyzes transfer of biotin to the epsilon amino group of a specific lysine residue of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. Sequences of naturally biotinylated substrates are highly conserved across evolutionary boundaries, and cross-species biotinylation has been demonstrated in several systems. To define the minimal substrate requirements in BirA-catalyzed biotinylation, we have measured the kinetics of modification of a 23-residue peptide previously identified by combinatorial methods. Although the sequence of the peptide bears little resemblance to the biotinylated sequence in BCCP, it is enzymatically biotinylated in vivo. Rates of biotin transfer to the 23-residue peptide are similar to those determined for BCCP. To further elucidate the sequence requirements for biotinylation, transient kinetic measurements were performed on a series of amino- and carboxy-terminal truncations of the 23-mer. The results, determined by stopped-flow fluorescence, allowed identification of a 14-residue peptide as the minimum required sequence. Additional support was obtained using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of peptides that had been incubated with an excess of biotinyl-5′-adenylate intermediate and catalytic amounts of BirA. Results of these measurements indicate that while kinetically inactive truncations showed no significant shift in molecular mass to the values expected for biotinylated species, kinetically active truncations exhibited 100% biotinylation. The specificity constant (k_{cat}/K_m) governing BirA-catalyzed biotinylation of the 14-mer minimal substrate is similar to that determined for the natural substrate, BCCP. We conclude that the 14-mer peptide efficiently mimics the biotin acceptor function of the much larger protein domain normally recognized by BirA.

Keywords: biotinylation; peptide mimic; post-translational modification; recognition

Target specificity in post-translational modification of proteins is of great biological significance. The structural determinants of this specificity are, in some reactions such as phosphorylation of tyrosine residues in SH2 domain target sites (Songyang et al., 1995) and prenylation (Reiss et al., 1991), well understood and are localized to the amino acid sequences surrounding the modification sites. In other systems such as the G-protein–coupled receptors, the recognition site for kinase binding and the phosphorylation site are distinct (Palczewski et al., 1995). Biotin-dependent carboxylases are a class of enzymes that undergo post-translational modification in which the biotin moiety is covalently linked to a single lysine residue via an amide bond. This two-step reaction, which is summarized in the following equations, is catalyzed by a class of enzymes termed the biotin holoenzyme synthetases (BHS) (Lane et al., 1964).

$$\text{biotin} + \text{ATP} \rightarrow \text{biotinyl-5′-AMP} + \text{PPi} \quad (1)$$

$$\text{bio-5′-AMP} + \text{apocarboxylase} \rightarrow \text{holocarboxylase} + \text{AMP}. \quad (2)$$

In the first step, the enzyme catalyzes formation of an activated intermediate, biotinyl-5′-adenylate (bio-5′-AMP) from biotin and ATP. Subsequent nucleophilic attack at the activated carboxylate of biotin results in formation of the amide between the biotin moiety and the target lysine residue. The biotinylation reaction is highly specific, with only the biotin-dependent carboxylases serving as substrates in vivo (Fall, 1979; Chandler & Ballard, 1988). Despite this high selectivity, very little is known about the structural determinants of specific enzyme-catalyzed biotinylation.

Only one target for biotinylation exists in *Escherichia coli*, the biotin carboxyl carrier protein (BCCP) subunit of acetyl CoA carboxylase (Robinson et al., 1983). In addition to the BCCP, two additional subunits are found in this enzyme, the biotin carboxylase and the transcarboxylase. A schematic diagram of the functional cycle of acetyl CoA carboxylase is shown in Figure 1 (Knowles, 1989). As indicated in the figure, the newly assembled

Reprint requests to: Dorothy Beckett, Department of Chemistry and Biochemistry, College of Life Sciences, Chemistry Bldg. 091, University of Maryland, College Park, Maryland 20742-2021; e-mail: dorothy_BECKETT@umail.umd.edu.

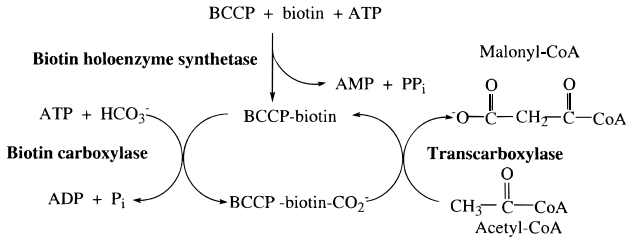


Fig. 1. Schematic outline of the functional cycle of the BCCP subunit of acetyl-CoA carboxylase. The BCCP is involved in three heterologous protein–protein interactions with the biotin holoenzyme synthetase (BirA), the biotin carboxylase, and the transcarboxylase.

enzyme first undergoes post-translational addition of biotin, a reaction catalyzed by the BHS (BirA). The biotin carboxylase subunit catalyzes transfer of CO_2^- from bicarbonate to a nitrogen of the ureido group of the BCCP-linked biotin moiety. Finally, the transcarboxylase subunit catalyzes transfer of the carboxylate to acetyl CoA to form malonyl CoA. All biotin-dependent carboxylases possess these three activities. While in prokaryotes they are found in three distinct subunits, in eukaryotes the multiple activities can be encoded on a single polypeptide chain.

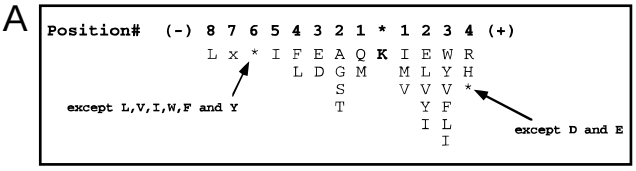
BHS-catalyzed addition of biotin is highly conserved across evolution. For example, the *E. coli* enzyme has been shown to biotinylate target substrates derived from organisms ranging from other bacteria to plants and humans (Cronan, 1990; Leon-Del-Rio & Gravel, 1994). The human BHS has, furthermore, been demonstrated to rescue a temperature sensitive mutant of BHS in *E. coli* (Leon-Del-Rio et al., 1995). Examination of the primary sequences of biotinylated subunits derived from a range of organisms indicates some sequence homology, particularly in the region of target lysine residue (Fig. 2) (Samols et al., 1988). Moreover, comparison of sequences of the BHSs from five organisms that have thus far been determined reveals a considerable degree of conservation, particularly in regions that are thought to form the enzyme active site (Tissot et al., 1997). The three-dimensional structure of a C-terminal domain fragment of the BCCP subunit from *E. coli* Acetyl CoA carboxylase has been determined in both its apo- and holo-forms by NMR spectroscopy and X-ray crystallography, respectively (Athapilly & Hendrickson, 1995; Yao et al., 1997). The fragment is functionally indistinguishable from the intact BCCP subunit in BHS-catalyzed biotinylation (Nenortas & Beckett, 1996). In these structures the target lysine residue is found in a β-turn and is accessible to the BHS for biotinylation. Based on the high degree of primary structure conservation observed among proteins of this class, all presumably fold into structures similar to that of BCCP (Brocklehurst & Perham, 1993). The structure of the BHS from *E. coli* has also been determined (Wilson et al., 1992).

Limited studies of the significance of the conserved sequences in biotinylated domains for selective post-translational modification have been performed. Results of these studies are inconclusive. For example, mutation of either conserved methionine adjacent to the target lysine residue in the *Propionibacterium shermanii* 1.3S subunit of transcarboxylase has no effect on biotinylation (Shenoy et al., 1988). Rather, these mutations were shown to function in carboxylate transfer to and from the biotin moiety (Shenoy et al., 1992). Deletion of conserved hydrophobic residues at the C-terminus of the protein 35 residues away from the biotinylated lysine does result in loss of biotinylation activity (Murtif & Samols, 1987). These results illustrate a fundamental difficulty associated with relating structure to function in a protein that participates in multiple interactions. As indicated in Figure 1, the BCCP of any biotin-dependent carboxylase is involved in three distinct heterologous protein–protein interactions (Knowles, 1989). First, the biotin holoenzyme synthetase must selectively interact with the protein in the biotin transfer reaction. Second, the biotin carboxylase must interact with bioB-CCP in the carboxylation reaction. Finally, the transcarboxylase interacts with the carboxybiotinylated BCCP in transfer of CO_2 to acetyl CoA. Any sequence conservation among these biotinylation targets thus reflects the products of multiple evolutionary constraints. Consequently, prediction of the significance of the sequence conservation for any one of the functions is difficult.

The issue of substrate selectivity in a biochemical reaction can also be approached using the powerful combinatorial methods that have been recently developed. One of these approaches involves screening of potential target ligands using large libraries of peptides linked to the terminus of *E. coli* lac repressor (Cull et al., 1992). This method has been utilized to screen peptide libraries for activity in BirA-catalyzed biotinylation in *E. coli* (Schatz, 1993). Results of screens of four biased peptide libraries has led to the identification of a "consensus peptide" for biotinylation (Fig. 3), the sequence of which bears little similarity to the known biotinylated sequences of BCCP subunits. The identified sequences have been fused to either the N-terminus or the C-terminus of a variety of proteins, and can be biotinylated either in vitro or in vivo (Altman et al., 1996; Kim & McHenry, 1996; Tatsumi et al., 1996; Tsao et al., 1996; Smith et al., 1998). These previous results,

• H.sapien PC	..PGKVIDIKVVAGAKVAKGQPLCVLSAM**K**METVVTSPMEGTV..
• H.sapien PCC	..PGVVVAVSVKPGDAVAEGQEICVIEAM**K**MQNSMTAGKTGTV..
• E.coli ACC	..PSPDAKAFIEVGQKVNVGNTLCIVEAAM**K**MMNQIEADKSGTV..
• Chicken ACC	..AGKLIQYVVEDGGHVFAGQCFAEIEVM**K**MVMTLTAGESGCI..
• Tomato	..VGLEVKVLVKDGEKVQEGQPLLVLEAM**K**MEHVVKAPANGYV..
• S.cerevisiae PC	..AGVIVEVKVHKGSLIKKGQPVAVLSAM**K**MEMIISSPSDGQV..
• P.shermanii TC	..AGTVSKILVKEGDTVKAGQTVLVLEAM**K**METEINAPTDGKV..

Fig. 2. Sequences in the vicinity of the target lysine residue (**K**) of BCC domains from several organisms are highly conserved. PC, pyruvate carboxylase; PCC, proprionyl-CoA carboxylase; ACC, acetyl-CoA carboxylase; TC, transcarboxylase.



B
#45 LGGIFEAM**K**MELRD
#37 LFLHDFLNAQ**K**VELYPVTSSG
#85 MAGGLNDIFEAQ**K**IEWHEDTGGS

Fig. 3. A: Summary of amino acid sequences active in BHS-catalyzed biotinylation of peptide substrates obtained from screens of four biased combinatorial libraries (Schatz, 1993). The bold **K** is the target lysine residue, and was present in all sequences. Numbering system: (−) residues N-terminal to **K**, (+) residues C-terminal to **K**. For positions in which several residues are shown, peptides in which any of those amino acids were in those positions were active in the screen. The "consensus sequences" for biotinylation do not reflect absolute sequence requirements for function. **B:** Sequences of peptides 37, 45, and 85.

however, provide no quantitative information on the efficiency with which these peptides are biotinylated. For example, although the results of the screening indicate that a number of peptide sequences can be biotinylated, some sequences may be better substrates than others. It is, furthermore, important to determine how the peptides compare to the natural substrate, apoBCCP, as biotinylation targets. Results of these quantitative measurements may allow identification of peptides that serve as kinetic mimics for the natural substrate. These peptide mimics will not only prove useful in elucidating structure–function relationships in selective biotinylation, but will also provide tools with multiple applications in protein chemistry and cell biology.

In this work we have characterized BirA-catalyzed biotin transfer to a number of peptide substrates. A fluorescence-based stopped-flow method for determination of initial rates of BirA-catalyzed biotinylation of BCCP has been developed in one of our laboratories (D.B.). This method has been used to characterize the kinetics of the second step of the reaction shown in Equations 1 and 2 (Nenortas & Beckett, 1996), transfer of biotin from the intermediate bio-5′-AMP. In this work, these initial rate measurements have been combined with an equilibrium method to quantitatively characterize the function of the peptide substrates in BirA-catalyzed biotinylation. Results of these measurements indicate that, in the context of a maltose-binding protein fusion, the peptides exhibit a range of activities in the biotinylation reaction. These initial measurements allowed identification of a 23-residue peptide, which, when fused to the N-terminus of MBP, is identical to the natural BCCP substrate in the biotinylation reaction. On its own, this same peptide is also identical to the natural substrate in BirA-catalyzed modification. Measurements of biotinylation of a series of truncates of the 23-mer have allowed identification of a 14-residue minimal substrate. The specificity constant (k_{cat}/K_m) governing BirA-catalyzed biotinylation of this peptide is nearly identical to that measured for the natural protein substrate apoBCCP.

Results

As discussed in the introduction, a number of peptides that serve as substrates for BirA-catalyzed biotinylation have been identified from screens of four biased peptide libraries fused to the lac repressor (Schatz, 1993). The results of these studies are, however, qualitative in nature and it is of interest to obtain a quantitative measures of activities of these peptide sequences in post-translational biotin addition. Therefore, three maltose binding fusion proteins containing peptides 37, 45, and 85 from the original screen were subjected to further analysis (Fig. 3B). Each of these peptides resulted from screens of separate libraries. Peptide 45 was obtained from a library, the sequence of which was biased to resemble that of the natural biotin acceptor proteins, peptide 37 was obtained from a library that was, with the exception of the biotinylated lysine residue, completely random, and peptide 85 was obtained from a library that was biased to fit the consensus sequence arrived at from screening four other peptide libraries. As the "consensus sequence" shown in Figure 3A does not represent absolute sequence requirements for biotinylation but merely a consensus of the peptide libraries screened, the presence of apparently noncompatible residues in peptide 37 is not inconsistent with activity in BHS-catalyzed biotin transfer. The fusion proteins were purified using an amylose affinity resin to which MBP binds. To prepare a homogeneous substrate for BirA assays, the unbiotinylated fraction of these proteins was purified by depleting the biotinylated fraction using streptavidin–agarose columns. These purified fusion proteins were biotinylated in vitro using BirA, and the relative levels of biotinylation were examined by immunoblotting. The BirA-treated proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with streptavidin–alkaline phosphatase conjugate. All of the proteins were detectably biotinylated in vitro by BirA, although the level of biotinylation of the peptide 85 derivative was significantly higher than that of the peptide 37 or peptide 45 fusion proteins (data not shown). These same three fusion proteins were subjected to kinetic measurements of BirA-catalyzed biotinylation.

The two-step biotinylation reaction is shown in Equations 1 and 2. Measurements of the initial rate of BirA-catalyzed transfer of biotin from bio-5′-AMP to the acceptor protein can be performed using stopped-flow fluorescence (Nenortas & Beckett, 1996). This measurement is based on the fact that binding of the intermediate in the biotin transfer reaction, bio-5′-AMP, results in a 40% quenching of the intrinsic fluorescence emission spectrum of BirA. Binding of the biotin acceptor protein, BCCP, followed by chemical transfer of biotin, is accompanied by a restoration of the fluorescence spectrum of unliganded BirA. The time course of transfer can, thus, be measured by mixing a complex of BirA-bio-5′-AMP with the acceptor protein and monitoring the resulting time-dependent increase in BirA fluorescence. A complication associated with performing such measurements on the fusion proteins arises from the fact that the maltose binding protein contains eight tryptophan residues per polypeptide chain (Duplay et al., 1984). Because the initial rate measurements are performed under pseudo-first-order conditions ([acceptor] $\gg$ [BirA]), a significant fluorescence contribution arises from the substrate. This contribution from the substrate interferes with measurement of the signal from BirA. To avoid this technical difficulty, a derivative of BirA in which tryptophan is replaced by 5-OH tryptophan was utilized for the measurements. The advantage of using this derivative is that its excitation spectrum extends out to 320 nm (Ross et al., 1992). One can thus excite the fluorescence of the 5-OH trp BirA in the presence of a large quantity of trp-containing acceptor protein without interference from this second protein. The time courses of biotin transfer obtained with BCCP87 and the peptide 85 derivative are shown in Figure 4. BCCP87 refers to an 87-residue C-terminal fragment of BCCP that has previously been shown to be functionally identical in biotin transfer to the intact protein (Nenortas & Beckett, 1996). Although the traces obtained with the peptide fusion protein substrate reproducibly exhibit an initial drop in the fluorescence signal, the subsequent time-dependent increase in fluorescence is well described by a single exponential model. [This initial drop in fluorescence intensity is observed only with the 5-OH tryptophan-labeled enzyme and peptide, either alone (see below) or MBP-fusions, substrates. The time scale of the drop (5 s) is much greater than the dead time of the instrument (5 ms). In analyzing the data for biotin transfer kinetics, only the single exponential increase in fluorescence intensity was considered.] Moreover, the apparent first-order rates determined from the time courses at 10 μM substrate are 0.107 ± 0.001 s^{-1} and 0.112 ± 0.002 s^{-1} for BCCP87 and the peptide 85 MBP fusion protein, respectively. This substrate concentration is within the linear range ($[S] < K_m$) of the initial rate vs. substrate concentration curve (Nenortas & Beckett, 1996). By contrast, peptide 37 and 45 fusion proteins exhibited no activity in this assay, suggesting that rates of biotin transfer to these peptides fused to MBP are much slower than those measured for the natural substrate or to the peptide 85 fusion.

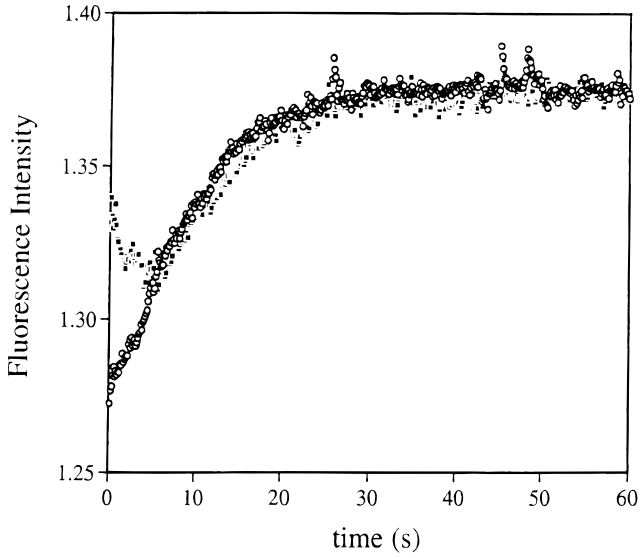


Fig. 4. Stopped-flow time courses obtained from measurements of biotin transfer to ○-BCCP87 and ■-MBP-peptide 85 fusion. The measurement was performed in standard buffer at 20 °C by rapidly mixing a solution containing 5-OH-trp-BirA + bio-5′AMP with a solution of the acceptor. Final concentrations—5-OH-trp-BirA (1 μM), bio-5′-AMP (0.9 μM), acceptor (10 μM). The excitation wavelength was 316 nm, and emission was monitored above 340 nm using a cutoff filter.

Peptide substrates in BirA-catalyzed biotin transfer

Results of kinetic measurements described above indicate that the N-terminal maltose binding protein fusion of peptide 85 is, at limited substrate concentration, identical to the natural substrate in BirA-catalyzed biotin transfer. This peptide, consequently, was subjected to further analysis. To determine whether this activity is due to the peptide sequence alone, the 23-residue peptide 85 was chemically synthesized, and the rates of BirA-catalyzed transfer of biotin to the substrate were measured over a range of peptide concentrations. This concentration range is in the linear region of the apparent rate versus substrate concentration curve. The presence of only a single tryptophan in the peptide sequence allowed use of the unlabeled BirA for the measurements. The apparent rates of biotin transfer to this peptide substrate are identical to those measured for the fusion protein and the natural substrate over a similar substrate concentration range (Table 1).

Table 1. *Apparent rates of BirA-catalyzed biotin transfer to peptide 85 and BCCP87*[a]

[Substrate] ($\times 10^6$)[b]	Peptide 85 (s^{-1})	BCCP87 (s^{-1})
10	0.086 ± 0.003	0.091 ± 0.003
15	0.126 ± 0.004	0.115 ± 0.003
20	0.17 ± 0.01	0.148 ± 0.005

[a]Measurements were made using stopped-flow fluorescence as described in Materials and methods. The final concentration of the BirA-bio-5′-AMP complex was 0.9 μM for all determinations.
[b]Final substrate concentrations after mixing.

To establish the minimal target for BirA-catalyzed biotinylation, a series of truncations of peptide 85 were synthesized and the apparent first-order rates of biotinylation of the peptides were determined by stopped-flow fluorescence (Fig. 5). Representative traces obtained in these measurements are shown in Figure 6, and a summary of the results obtained with all peptides is shown in Figure 7. These results indicate that the rates of biotin transfer are nearly identical for all amino terminal truncations of peptide 85 up to position −9 relative to the biotinylated lysine residue. The measured rates of biotin transfer to peptides in the N-terminal truncation series drops precipitously with the removal of the G at position −9. In the C-terminal truncation series, the three peptides corresponding to progressive deletion of residues up to position +7 relative to the modified lysine all exhibit similar rates of BirA-catalyzed biotinylation. Deletion of the D at position +6 yields a substrate that is reproducibly biotinylated at a higher rate that any other peptide or the natural substrate. Further truncation of residues in this series leads to gradual loss of activity in the kinetic assay.

The observed loss of activity in the stopped-flow based kinetic assay does not imply that these peptides are not biotinylated at all in the BirA-catalyzed reaction. To determine if peptides that are determined to be "poor" substrates in the kinetic assay are biotinylated to any extent as well as to establish that kinetically "good" peptide substrates are converted to the masses consistent with addition of the biotin moiety, experiments designed to measure equilibrium levels of biotinylation were performed. These measurements were performed by combining the peptides with a catalytic amount of BirA and an excess of chemically synthesized bio-5′-AMP. The reactions were allowed to reach equilibrium and the products were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis. Representative spectra are shown in Figure 8, and a summary

Intact Peptide

85-1 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S

Amino-terminal Truncation Series

85-2 A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S
85-3 G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S
85-4 G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S
85-5 L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S
85-6 N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G-S

Carboxy-terminal Truncation Series

85-7 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G-G
85-8 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T-G
85-9 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D-T
85-10 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E-D
85-11 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H-E
85-12 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H
85-13 M-A-G-G-L-N-D-I-F-E-A-Q-**K**-I-E-W

Minimal Substrate

85-14 G-L-N-D-I-F-E-A-Q-**K**-I-E-W-H

Fig. 5. Sequences of the truncation series of peptide 85. The amino-terminal and carboxy-terminal series represent successive deletion of residues from the amino- and carboxy-termini, respectively. The double truncate was predicted, based on results of measurements of biotin transfer to the N-terminal and C-terminal truncation series, to be the minimal peptide mimic of the natural substrate.

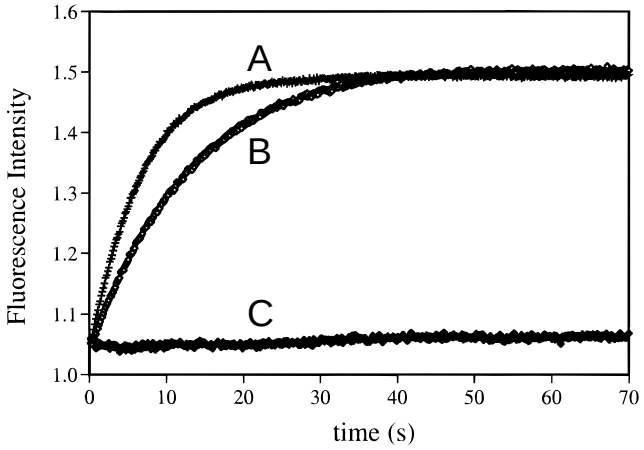


Fig. 6. Stopped-flow traces obtained from measurements of the time course of BirA-catalyzed transfer of biotin from bio-5′-AMP to substrates (**A**) BCCP87, (**B**) peptide 85-14, and (**C**) peptide 85-6. In all measurements, the final peptide concentration was 20 μM and the BirA and bio-5′-AMP concentrations were as indicated in Figure 4. The excitation wavelength was 295 nm, and emission was monitored above 340 nm using a cutoff filter. All measurements were performed in standard buffer at 20 °C.

of the results obtained for all peptides in the series is shown in Table 2. The measured masses of the biotinylated and unmodified peptides are in excellent agreement with masses calculated from composition. Furthermore, the results of these equilibrium measurements of biotinylation are consistent with the results of kinetic measurements described above. All peptides that are biotinylated at rates comparable to that of the natural substrate, BCCP, are also quantitatively converted to the biotinylated species in this assay. Those peptides that exhibit very low or unmeasurable rates of biotinylation in the kinetic assay are converted to the biotinylated species at very low levels in the equilibrium assay.

A minimal peptide substrate in BirA-catalyzed biotinylation

Based on the results obtained with the peptide-85 truncation series, the minimal predicted substrate in BirA-catalyzed biotinylation is 14 residues long (Fig. 5). This peptide was tested in the steady-state biotinylation assay and, as predicted, was quantitatively converted to the biotinylated form (Table 2). The peptide was also tested in kinetic measurements to determine the specificity constant for biotin transfer. Because these measurements required extending the substrate concentration to the 100 μM range at which the tryptophan absorbance and fluorescence signals associated with the substrate interferes with the spectral signals associated with the enzyme, the 5-OH tryptophan derivative of BirA was utilized for the determination. The dependence on substrate concentration of the pseudo-first-order rate of biotin transfer to the natural substrate, BCCP87 and to the 14-mer are shown in Figure 9. The values of k_{cat}/K_m for the two substrates estimated from nonlinear least-squares analysis of the data are 11,900 ± 400 M^{-1} s^{-1} for BCCP vs. 10,000 ± 500 for peptide 85–14. These results are notable at two levels. First, the value of k_{cat}/K_m determined for the natural substrate using 5-OH trp BirA is in very good agreement with the previously published value of 14,700 ± 300 M^{-1} s^{-1} determined using the unlabeled BirA enzyme (Nenortas & Beckett, 1996). Second, the estimated k_{cat}/K_m governing BirA-catalyzed biotinylation of the 14-residue peptide is nearly identical to the value measured for the natural substrate.

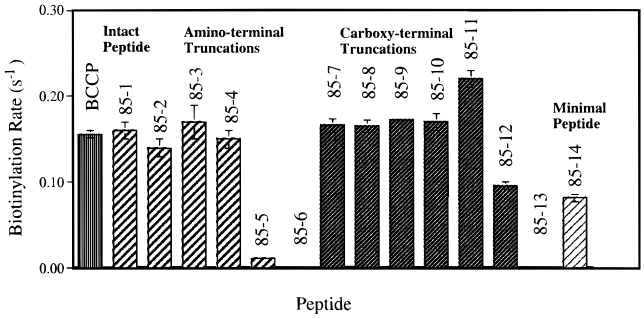


Fig. 7. Summary of the results of measurements of the initial rates of BirA-catalyzed biotination of the peptide 85 truncation series. All measurements were performed as indicated in Figure 6. The reported rate is the average of five to seven stopped-flow traces and error bars represent the standard deviation.

Table 2. *Mass spectrometric analysis of equilibrium levels of modification of peptide substrates in BirA-catalyzed biotination*[a]

Peptide no.	Unbiotinylated mass (Da)		Biotinylated mass (Da)		Fraction modified[c]
	Calculated	Measured	Calculated	Measured[b]	
85-1	2,506	2,506 ± 5	2,732	2,733 ± 4	1.00
85-2	2,376	2,375 ± 3	2,602	2,602 ± 3	1.00
85-3	2,304	2,305 ± 4	2,530	2,529 ± 3	1.00
85-4	2,246	2,247 ± 4	2,472	2,475 ± 3	1.00
85-5	2,190	2,188 ± 4	2,416	2,417 ± 3	0.70
85-6	2,076	2,077 ± 2	2,302	2,078 ± 3	~0
85-7	2,419	2,419 ± 3	2,645	2,650 ± 4	1.00
85-8	2,362	2,363 ± 2	2,588	2,589 ± 6	1.00
85-9	2,305	2,305 ± 3	2,531	2,531 ± 3	1.00
85-10	2,203	2,204 ± 4	2,429	2,430 ± 5	1.00
85-11	2,089	2,089 ± 2	2,315	2,317 ± 2	0.95
85-12	1,958	1,963 ± 1	2,184	2,189 ± 3	1.00
85-13	1,822	1,824 ± 3	2,048	1,825 ± 4	0.10
85-14	1,700	1,703 ± 3	1,926	1,928 ± 2	1.00

[a] Reactions were performed in standard buffer for 1 h at 20 °C and contained 100 μM peptide substrate, 200 μM bio-5′-AMP, and 1 μM BirA. Samples were prepared for MALDI-TOF analysis as described in Materials and methods.

[b] The measured masses that are identical to those expected for the unbiotinylated peptides were measured for only those peptides that were biotinylated at very low levels (<0.1).

[c] The fraction modified was estimated from the relative areas of the peaks corresponding to the unbiotinylated and biotinylated species.

Function of the minimal substrate in a maltose binding protein fusion

To measure the properties of the minimal peptide substrate in BHS-catalyzed biotinylation in the context of a large protein, a gene encoding a fusion of the peptide to the C-terminus of maltose

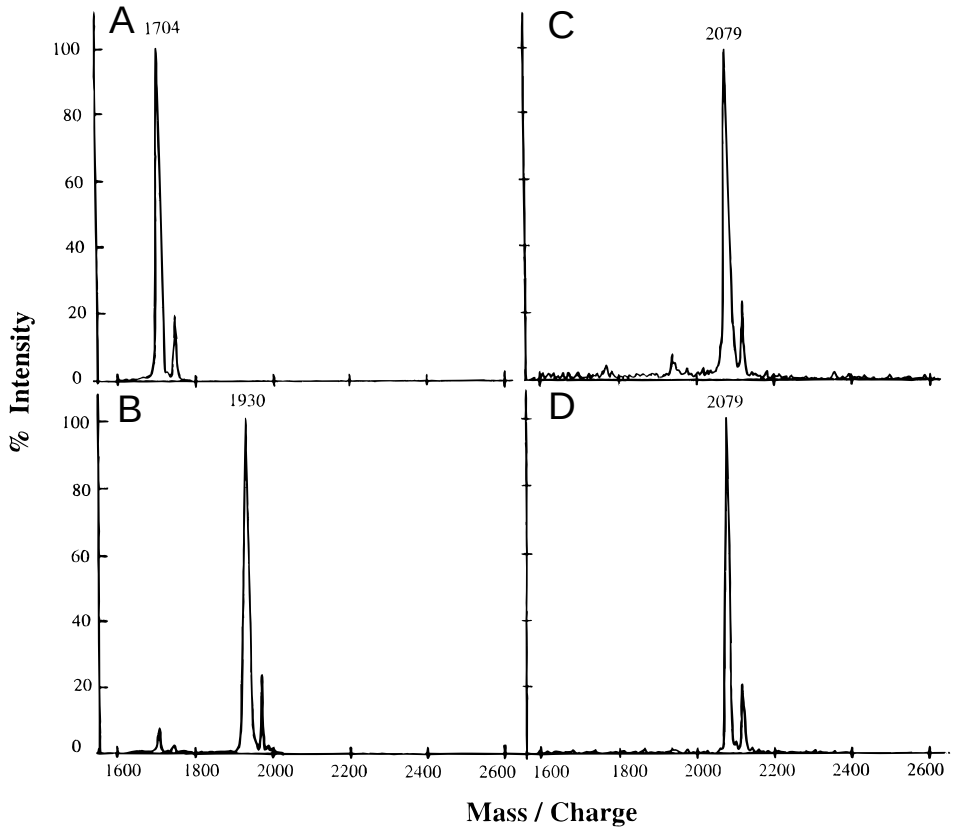


Fig. 8. MALDI-TOF spectra obtained for peptides 85-14 and 85-6. Spectra of samples obtained for the (**A, C**) unbiotinated and (**B, D**) biotinated peptides are shown. Unbiotinylated refers to peptides that were incubated with an excess of biotin in the absence of the BHS. Biotinylated refers to peptides that had been incubated with both an excess of the adenylate and the enzyme. Samples were prepared for MALDI-TOF as described in Materials and methods.

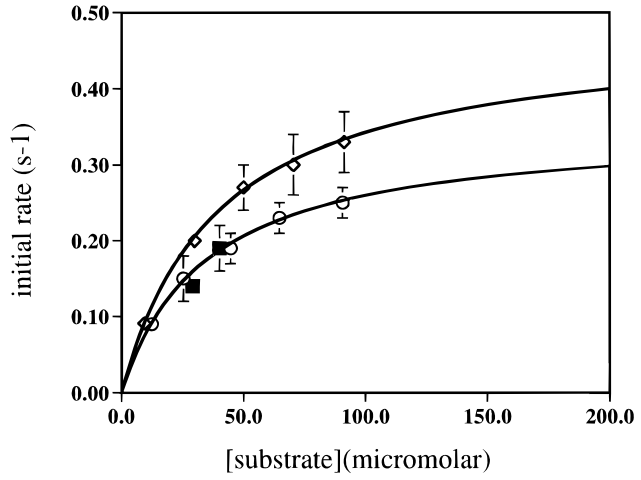


Fig. 9. Measurements of the dependence of the pseudo-first-order rate of 5-OH-trp-BirA-catalyzed biotin transfer on substrate concentration. BCCP87-◇, Peptide 85-14-○, and MBP-14-mer-■. The reactions were carried out as described in Figure 5 with the exception that substrate concentration was varied. The solid lines were obtained from simulation of the hyperbolic curve using values of the kinetic parameters obtained from fitting the data directly to the Michaelis–Menten equation.

binding protein was constructed, and the protein was overexpressed and purified. This fusion was designed with a TGG linker between the target peptide and the C-terminus of the MBP (after the Factor Xa site encoded by the pMAL-c2 vector sold by New England Biolabs, Beverly, Massachusetts). Initial in vivo measurements indicated that the protein is biotinylated in *E. coli*. Initial rates of BirA-catalyzed biotinylation of the fusion protein were measured over a limited range of substrate concentration using the 5-OH trp BirA derivative, results of which are shown in Figure 9. These results indicate that the peptide activity in biotinylation in the context of a protein is indistinguishable from that measured for the free peptide, and that the core peptide 85 sequence allows efficient biotinylation when fused at the C-terminus of a target protein.

Discussion

The post-translational addition of biotin to biotin-dependent carboxylases is highly selective in vivo. Despite a wealth of sequence information available for biotinylated subunits from a range of organisms, as well as the knowledge of the three-dimensional structures of two members of this class of proteins (Athappilly & Hendrickson, 1995; Yao et al., 1997; Reddy et al., 1998), there is currently little understanding of the relationship between structure

and selectivity in the biotinylation reaction (Shenoy et al., 1988). It has been demonstrated, from studies of the 1.3S subunit of transcarboxylase from *P. shermanii*, that some highly conserved residues in the vicinity of the target lysine residue are not required for selective biotinylation (Murtif & Samols, 1987). In this same protein, mutation of hydrophobic residues at the carboxy terminus prevents biotinylation, despite the fact that these residues are far removed from the target lysine in the folded protein. Comparison of BCC sequences indicates that these residues are conserved across the evolutionary spectrum. Results of studies of sequence requirements for biotinylation of the biotin-accepting domain of human propionyl CoA carboxylase, however, indicate that analogous residues at the C-terminus of this protein are not significant for biotinylation (Leon-Del-Rio & Gravel, 1994). Several other mutations resulted in loss of biotinylation activity in this latter protein, but it is difficult to distinguish this apparent loss of activity from folding defects. In summary, a large gap exists between our knowledge of structure and our understanding of the significance of that structure for activity in selective post-translational addition of biotin.

The combinatorial approach used to identify peptide sequences that are biotinylated in vivo yielded information to propose a consensus biotinylation site shown in Figure 3. Interestingly, this sequence bears little resemblance to naturally biotinylated sequences (Schatz, 1993). The identification of this consensus sequence was based only on the results of in vivo studies of the combinatorial libraries. In this work we have used biochemical techniques to quantitatively characterize the peptide sequence identified from the combinatorial libraries. Results of initial studies of representative peptides from three of the libraries revealed a range of activities in the biotinylation assay. MBP fusion proteins of peptides 45 and 37 both show no activity in measurements of initial rates of BirA-catalyzed transfer of biotin from bio-5′-AMP. Because results of in vivo and in vitro studies indicate that fusion proteins of these peptides are biotinylated, we conclude that their rates of biotinylation are considerably slower than that measured for the natural substrate. Interestingly, while peptide 45 was obtained from a library for which the sequence was biased to resemble that of the natural biotin acceptor proteins, peptide 37 was obtained from a library that was, with the exception of the biotinylated lysine residue, completely random. The maltose binding protein fusion of peptide 85 as well as peptide 85 alone exhibit kinetics identical to the natural BCCP substrate in BirA-catalyzed biotinylation. This peptide was obtained from a library that was biased to fit the consensus sequence arrived at from screening four other peptide libraries. Thus, it is not surprising that, of the three peptides tested, it is the most active in the biotinylation assay.

In evaluating the biotin acceptor activities of the peptides in the context of a fusion protein the potential influence of the maltose binding protein sequences must be considered. For example, although of the first three fusion proteins examined in this study the peptide 85 sequence was by far the best substrate, it is possible the result does not solely reflect the properties of the peptide. This is because, of the three, the peptide 85 fusion was the only N-terminal fusion examined and BirA preferentially recognizes peptides fused at the N-terminus of MBP. However, the observations that peptide 85 is an equivalent substrate alone and when fused to the N-terminus of MBP and that the 14-mer, which contains the peptide 85 core sequence, is also an equivalent substrate alone or when fused at the C-terminus of MBP renders this explanation unlikely. Therefore, although we have not measured the activities of the other two peptides alone, we conclude that the peptide 85 sequence is an intrinsically better substrate for BirA than peptides 37 or 45.

The 23-residue peptide, peptide 85, was used as a starting point for defining a minimal peptide substrate in BirA-catalyzed biotinylation. Analysis of a truncation series of the original peptide has yielded a 14-residue peptide that is biotinylated with a specificity constant similar to that measured for the natural substrate. The sequence of this peptide bears little resemblance to that of the naturally biotinylated substrate. Only four residues, at positions -2, -3, -5, and $+2$ relative to the target lysine residue in the peptide, are also found with relatively high frequency in the natural substrate. For example, hydrophobic residues are found with high frequency at position -5 in natural targets. An L is located at this position in the minimal peptide. Moreover, consistent with the natural substrate in which residues at positions -3 and -2 have a high likelihood to be E or A, respectively, these same residues are found in the minimal substrate. Finally, as observed in many natural substrates, E is found at position $+2$ in the peptide sequence. As indicated in the introduction, the structure of the C-terminal domain of BCCP has been determined by both X-ray crystallography and NMR spectroscopy. In this structure, the four residues indicated above all lie on the same face of the protein surface that is created by the β-turn in which the target lysine is found. Although results of circular dichroism measurements indicate that the 14-residue peptide is unstructured on its own (Beckett et al., unpubl. obs.), it is possible that when bound to BirA the peptide adopts a structure that presents a surface similar to that identified in BCCP. Peptides 37 and 45, which also contain this same four residue pattern in the vicinity of the target lysine residue, may be biotinylated at lower rates than peptide 85 because they do not readily adopt the structure that is required for their recognition by BirA.

The availability of a peptide that is efficiently biotinated by BirA provides a tool with many potential applications. Due to the high affinity of the avidin– or streptavidin–biotin interaction, construction of fusions of this peptide to any protein yields a tag for purification, immobilization, or extremely sensitive detection. Furthermore, the small size of the peptide decreases the probability of its interfering with the function of the protein to which it is fused. Although the 14-mer is within twofold of any other substrate in terms of catalytic efficiency, it is very likely that a 15-mer, based on the C-terminal sequence of peptide 85-11 (Fig. 5), would be a better fusion peptide, due to its consistently higher biotinylation rate (Fig. 7). We, therefore, recommend the 15-mer GLNDIFEAQK IEWHE as an optimal fusion partner for most applications of this biotinylation method. When fused at the N-terminus of a target protein, the sequence would obviously need to be preceded by a methionine start codon along with a second codon consistent with efficient translation and protein stability. For example, the starting sequence MA, encoded by the base sequence ATGGCT, should be efficiently expressed in *E. coli*. At the C-terminus of a fusion protein, the 15-mer above is generally connected to the protein through, at minimum, a GG peptide linker.

Although the biotin holoenzyme synthetase recognition reaction is extremely conserved among a variety of species, the ability of these peptides to be recognized by BHS enzymes other than *E. coli* BirA has not been tested. It will be interesting to determine whether the peptides mimic a conserved structure that might be widely recognized, or alternatively, whether they are only recognized by BHS enzymes similar to BirA.

Materials and methods

Chemicals and biochemicals

All chemicals used in buffer preparation were at least reagent grade. The d-biotin was purchased from Sigma (St. Louis, Missouri). BirA was purified as described in Abbott and Beckett (1993), and its concentration was determined by UV absorbance using an extinction coefficient at 280 nm of 1.3 $(mg/mL)^{-1}$ cm^{-1}. Bio-5′-AMP was synthesized using a modification of the method described in Lane et al. (1964). The compound was stored desiccated at −20 °C. The dry powder was diluted in dH_2O for use in experiments, and these solutions were stored at −70 °C. BCCP87 was purified as described in Nenortas and Beckett (1996). Its concentration was determined by UV absorbance using an extinction coefficient of 1,450 L/mol cm at 276 nm. The L-tryptophan and L-5-OH tryptophan were purchased from Sigma.

Preparation of 5-OH tryptophan-labeled BirA

The 5-OH-tryptophan analog was introduced into BirA using a modification of the in vivo labeling procedure described in Ross et al. (1992). The protein was overexpressed from the plasmid pJMR1 (a gift from Dr. Anthony Otsuka) in the tryptophan auxotroph CY15077 (constructed in the laboratory of Dr. Charles Yanofsky). Because transcription of the *birA* gene from this plasmid is under control of the *tac* promoter, the strain also contained the *lacI*-encoding plasmid pMS421. The resulting strain is ampicillin and streptomycin resistant. For protein overexpression the cells were grown in M9 medium supplemented with 0.25 mM L-tryptophan (Sigma), and the appropriate antibiotics until the culture reached an optical density of 1.2 at 600 nm. At this point the cells were pelleted by centrifugation at 4,500 rpm for 15 min at 4 °C. The cell pellet was resuspended in 2 L of M9 medium containing ampicillin and streptomycin and no tryptophan. This cellular suspension was allowed to incubate for 1 h, at which point 5-OH tryptophan was added to a final concentration of 0.25 mM. After incubation for an additional hour at 37 °C transcription of the *birA* gene was induced by addition of IPTG to a final concentration of 75 μg/mL. Induction was allowed to proceed for 4 h, at which point the cells were harvested by centrifugation at 4,500 rpm for 15 min at 4 °C. The 5-OH–labeled protein was purified as described in Abbott and Beckett (1993). Approximately 10 mg of protein was obtained from 2 L of culture. The protein concentration was estimated by densitometry of a sample that had been subjected to SDS-PAGE and stained with Coomassie brilliant blue. A standard curve for this procedure was generated using samples of known concentration of unlabeled BirA as standards.

Preparation of maltose binding protein fusion proteins

E. coli cultures carrying plasmids expressing MBP fusion proteins #37, #45, and #85 (Schatz, 1993) were induced with 0.3 mM IPTG (#37 and #45) or with 0.2% L-arabinose (#85) for 2 h. MBP proteins were purified from the cells on amylose columns essentially as described by the manufacturer (New England Biolabs). Unbiotinylated fusion proteins were produced from the partially biotinylated MBP fusion proteins by depleting the biotinylated fraction using either streptavidin–agarose (Pierce, Rockford, Illinois) or avidin–agarose (Sigma).

Peptide synthesis and purification

All peptides were synthesized by SynPep Corporation (Dublin, California) or at Affymax (Palo Alto, California) using standard chemistry. The products were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C-18 column using a 0.1% TFA in H_2O to 0.075% TFA in acetonitrile gradient. The peptides were estimated to be 99% pure by HPLC, and all masses were confirmed by electrospray ionization mass spectrometry. Peptides were prepared for biotin transfer measurements by resuspending the dry powder in Standard Buffer (10 mM Tris-Base, 200 mM KCl, 2.5 mM $MgCl_2$) that had not been titrated. Aliquots of 0.3 M HCl were added to the resulting solution until pH 7.5 was obtained. The peptide concentrations were determined by UV absorbance using an extinction coefficient of 5,690 M^{-1} cm^{-1} at 280 nm. This extinction coefficient is based on the peptide composition of 1 tryptophan per peptide (Edelhoch, 1967). The small contribution of phenylalanine to the absorbance was not included in this estimate.

Stopped-flow measurements of initial rates of BirA-catalyzed biotin transfer

Stopped-flow fluorescence measurements were performed as described in Nenortas and Beckett (1996) using a KinTek model SF-2001 instrument equipped with a circulating water bath to maintain constant temperature. All measurements were performed in standard buffer adjusted to pH 7.50 ± 0.01 at 20.0 ± 0.1 °C. The measurements were performed by rapidly mixing a solution containing 2.0 μM BirA + 1.8 μM bio-5′-AMP with a solution of biotin acceptor (BCCP87, MBP fusion protein, or peptide) and monitoring the time-dependent increase in intrinsic BirA fluorescence that is associated with release of bio-5′-AMP. The excitation wavelength was set to 295 nm for all measurements in which unlabeled BirA served as the enzyme. For reactions in which 5-OH tryptophan-labeled BirA was utilized, the excitation wavelength was 316 nm. In all experiments the fluorescence emission was monitored above 340 nm using a cutoff filter.

Steady-state measurements of BirA-catalyzed biotinylation of peptides

Samples for measurements of equilibrium levels of post-translational modification of acceptor peptides were prepared by combining the acceptor peptide with a catalytic amount of BirA and a two- to fivefold excess of biotinyl-5′-AMP over peptide concentration in standard buffer. The reactions were allowed to proceed at 20 °C for 1 h. Control reactions in which no BirA was present were performed in parallel. Once the reactions were completed the mixtures were transferred to dialysis tubing (500 MW cutoff, Spectra/Por) and dialyzed extensively against 5% vol/vol acetic acid in H_2O. The dialyzed samples were transferred to 1.5 mL siliconized Eppendorf tubes and lyophilized to dryness. The lyophilized samples were dissolved in dH_2O to a final peptide concentration of 20 μM.

Matrix-assisted laser desorption time of flight mass spectrometry

Samples for MALDI-TOF were prepared by spotting 0.3 μL of each peptide solution and 0.3 μL 50 mM α-cyano 4-hydroxy cinnamic acid (in 80% vol/vol acetonitrile in 0.1% trifluoroacetic

acid) onto the sample slide. All spectra were acquired on a Kratos Kompact MALDI-TOF instrument (Shimadzu) operated in linear mode. The laser power ranged from 60–75. All masses of biotinylated and unbiotinylated peptides were measured at least in duplicate on independent samples.

Data analysis

Nonlinear least-squares analysis of the raw data from stopped-flow measurements was performed using either a single or double exponential model. The values of k_{cat}/K_m for the reaction were obtained from nonlinear least-squares analysis of the initial rate vs. substrate concentration profiles using the Michaelis–Menten equation.

Acknowledgments

The authors thank P. Balasubramanian, K. Yin, and S. Podduturi for synthesis and characterization of peptides, and E.D. Streaker for mass spectral measurements. This work was supported in part by NIH-GM46511 to D.B. The MALDI-TOF Mass Spectrometer was purchased using funds provided by a shared instrumentation grant NIH-RR08310 to Catherine Fenselau.

References

Abbott J, Beckett D. 1993. Cooperative binding of the *Escherichia coli* repressor of biotin biosynthesis to the biotin operator sequence. *Biochemistry 32*:9649–9656.

Altman JD, Moss PAH, Goulder JR, Barouch DH, McHeyzer-Williams M, Bell JI, McMichael AJ, Davis MM. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274*:94–96.

Athapilly FK, Hendrickson WA. 1995. Structure of the biotinyl domain of acetyl-coenzyme A carboxylase determined by MAD phasing. *Structure 3*:1407–1409.

Brocklehurst SM, Perham RN. 1993. Prediction of the three dimensional structures of the biotinylated domain from yeast pyruvate carboxylase of the lipoylated H-protein from the pea leaf glycine cleavage system: A new automated method for the prediction of protein tertiary structure. *Protein Sci 2*:626–639.

Chandler CS, Ballard FJ. 1988. Regulation of the breakdown rates of biotin-containing proteins in Swiss 3T3-L1 cells. *Biochem J 251*:749–755.

Cronan JE Jr. 1990. Biotinylation of proteins *in vivo*: A post-translational modification to label, purify and study proteins. *J Biol Chem 265*:10327–10333.

Cull MG, Miller F, Schatz PJ. 1992. Screening for receptor ligands using large libraries of peptides linked to the C terminus of lac repressor. *Proc Natl Acad Sci USA 89*:1865–1869.

Duplay P, Bedouelle H, Fowler A, Zabin I, Saurin W, Hofnung M. 1984. Sequences of the *malE* gene and of its product, the maltose-binding protein of *Escherichia coli* K12. *J Biol Chem 259*:10606–10613.

Edelhoch H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry 6*:1948–1954.

Fall RR. 1979. Analysis of microbial biotin proteins. *Methods Enzymol 62*:390–398.

Kim DR, McHenry CS. 1996. Biotin tagging deletion analysis of domain limits involved in protein macromolecular interactions. *J Biol Chem 271*:20690–20698.

Knowles JR. 1989 The mechanism of biotin-dependent enzymes. *Annu Rev Biochem 58*:195–221.

Lane MD, Rominger KL, Young DL, Lynen F. 1964. The enzymatic synthesis of holotranscarboxylase from apotranscarboxylase and (+) biotin. *J Biol Chem 239*:2865–2871.

Leon-Del-Rio A, Gravel RA. 1994. Sequence requirements for the biotinylation of carboxy-terminal fragments of human proprionyl-CoA carboxylase α subunit expressed in *Escherichia coli*. *J Biol Chem 269*:22964–22968.

Leon-Del-Rio A, Leclerc D, Akerman B, Wakamatsu N, Gravel RA. 1995. Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of *Escherichia coli*. *Proc Natl Acad Sci USA 92*:4626–4630.

Murtif VL, Samols D. 1987. Mutagenesis affecting the carboxyl terminus of the biotinyl subunit of transcarboxylase. *J Biol Chem 262*:11813–11816.

Nenortas E, Beckett D. 1996. Purification and characterization of intact and truncated forms of the *Escherichia coli* biotin carboxyl carrier subunit of acetyl-CoA carboxylase. *J Biol Chem 271*:7559–7567.

Palczewski K, Buczylko J, Kaplan MW, Polans AS, Crabb JW. 1995. Mechanism of rhodopsin kinase activation. *J Biol Chem 266*:12949–12955.

Reddy DV, Rothemund S, Shenoy BC, Carey PR, Sonnichsen FD. 1998. Structural characterization of the entire 1.3S subunit of transcarboxylase from *Proprionibacterium shermanii*. *Protein Sci 7*:2156–2163.

Reiss Y, Stradley SJ, Gierasch LM, Brown MS, Goldstein JL. 1991. Sequence requirement for peptide recognition by rat brain $p21^{ras}$ protein farnesyltransferase. *Proc Natl Acad Sci USA 88*:732–736.

Robinson BH, Oei J, Saunders M, Gravel R. 1983. ^{3}H-Biotin-labeled proteins in cultured human skin fibroblasts from patients with pyruvate carboxylase deficiency. *J Biol Chem 258*:6660–6664.

Ross JBA, Senear DF, Waxman E, Kombo BB, Rusinova E, Huang YT, Laws WR, Hasselbacher CA. 1992. Spectral enhancement of proteins: Biological incorporation and fluorescence characterization of 5-hydroxytryptophan in bacteriophage λ cI repressor. *Proc Natl Acad Sci USA 89*:12023–12027.

Samols D, Thornton CG, Murtif VL, Kumar GK, Haase FC, Wood HG. 1988. Evolutionary conservation among biotin enzymes. *J Biol Chem 263*:6461–6464.

Schatz PJ. 1993. Use of peptide libraries to map substrate specificity of a peptide-modifying enzyme: A 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology 11*:138–1143.

Shenoy BC, Paranjape S, Murtif VL, Kumar GK, Samols D, Wood HG. 1988. Effects of mutations at the Met-88 and Met-90 on the biotinylation of Lys-89 of the apo 1.3S subunit of transcarboxylase. *FASEB J 2*:2505–2511.

Shenoy BC, Xie Y, Park VL, Kumar GK, Beegan H, Wood HG, Samols D. 1992. The importance of methionine residues for the catalysis of the biotin enzyme, transcarboxylase. *J Biol Chem 267*:18407–18412.

Smith PA, Tripp BC, DiBlasio-Smith EA, Lu Z, LaVallie ER, McCoy JM. 1998. A plasmid expression system for quantitative *in vivo* biotinylation of thioredoxin fusion proteins in *Escherichia coli*. *Nucleic Acids Res 26*:1414–1420.

Songyang Z, Carraway KL III, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, Lorenzon MJ, Ponder BAJ, Mayer BJ, Cantley LC. 1995. Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature 373*:536–539.

Tatsumi H, Fukuda S, Kikuchi M, Koyama Y. 1996. Construction of biotinylated firefly luciferases using biotin acceptor peptides. *Anal Biochem 243*:176–180.

Tissot G, Douce R, Alban C. 1997. Evidence for multiple forms of biotin holocarboxylase synthetase in pea (*Pisum sativum*) and in *Arbidopsis taliana*: Subcellular fractionation studies and isolation of a cDNA clone. *Biochem J 323*:179–188.

Tsao K-L, DeBarbieri B, Michel H, Waugh DS. 1996. A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in *Escherichia coli*. *Gene 169*:59–64.

Wilson KS, Shewchuck LM, Brennan RG, Otsuka AJ, Matthews BW. 1992. *Escherichia coli* biotin holoenzyme synthetase/*bio* repressor crystal delineates the biotin- and DNA-binding domains. *Proc Natl Acad Sci USA 89*:9257–9261.

Yao X, Wei D, Soden C Jr, Summers MF, Beckett D. 1997 Structure of the carboxy-terminal fragment of the apo-biotin carboxyl carrier subunit of *Escherichia coli* acetyl-CoA carboxylase. *Biochemistry 36*:15089–1510.