Prokaryotic BirA ligase biotinylates K4, K9, K18 and K23 in histone H3†

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Abstract

BirA ligase, a prokaryotic ortholog of holocarboxylase synthetase (HCS), is known to biotinylate proteins. Here, we tested the hypothesis that BirA ligase catalyzes biotinylation of eukaryotic histones. If so, this would render recombinant BirA ligase a useful surrogate for HCS in studies of histone biotinylation. Biological activity of recombinant BirA ligase was confirmed by enzymatic biotinylation of p67. Importantly, BirA ligase biotinylated both calf thymus histone H1 and human bulk histone extracts. Incubation of recombinant BirA ligase with H3-based synthetic peptides revealed that lysines 4, 9, 18, and 23 in histone H3 are targets for biotinylation by BirA ligase. Modifications of peptides (e.g., serine phosphorylation) affected subsequent biotinylation by BirA ligase, suggesting crosstalk among modifications. In conclusion, this study suggests that prokaryotic BirA ligase is a promiscuous enzyme and biotinylates eukaryotic histones; biotinylation of histones by BirA ligase is consistent with the proposed role of human HCS in chromatin.

Keywords

biotin; BirA ligase; histones; holocarboxylase synthetase; p67

Introduction

Biotin serves as a coenzyme for five biotin-dependent carboxylases: acetyl-CoA carboxylases 1 and 2, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase (1). Binding of biotin to specific lysine (K) residues in carboxylases is mediated by holocarboxylase synthetase (HCS) (1-3). HCS was first purified from bovine liver cytosol and characterized by Chiba et al. (4). HCS has been detected in cytoplasm, mitochondria, and nuclei (5-8).

Prokaryotes such as Escherichia coli express an ortholog of human HCS: BirA ligase. BirA ligase mediates the binding of biotin to biotinyl carboxyl carrier protein, which is the bacterial counterpart of acetyl-CoA carboxylase (9). The biotinylation of apocarboxylases by both HCS and BirA ligase occurs by the addition of a biotin molecule to a specific lysine residue located in a conserved Met-Lys-Met domain (2,10).

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HCS and BirA ligase also share the following features which, theoretically, make BirA a good model for studies of protein biotinylation by HCS. BirA ligase plays a critical role in cell signaling and chromatin remodeling during the regulation of biotin biosynthesis in prokaryotes (11,12). Similar mechanisms of gene regulation have been discovered for HCS in eukaryotes. First, biotinyl-AMP appears to regulate the transcription of genes coding for propionyl-CoA carboxylase and pyruvate carboxylase in human liver cells by an unknown mechanism of action (13). Second, biotinylation of histones by HCS and biotinidase (BTD) (6,14) is associated with gene repression (8). For example, biotin promotes increased biotinylation of K12 in histone H4 (K12Bio H4) at promoter 1 of the sodium-dependent multivitamin transporter gene, repressing transporter transcription (15).

K12 in histone H4 is among several lysine residues on different histones that can be biotinylated in-vitro by BTD, and among several histone-specific biotinylation that have been shown to occur in-vivo (7,16,17). Notwithstanding the significance of these findings, it was subsequently suggested that HCS might be more important than BTD for biotinylating histones (6,18). For example, knockdown of HCS in Drosophila melanogaster decreased the abundance of biotinylated histones, affecting gene expression patterns and stress resistance in flies; in contrast, knockdown of BTD did not affect these variables to a large extent (18,19). Recombinant, bioactive HCS is difficult to produce because it typically does not fold correctly under standard conditions (unpublished observation). Here we tested the hypothesis that histones are targets for biotinylation by BirA ligase, and that BirA ligase is a useful surrogate for HCS in studies of histone biotinylation in vitro.

**Materials and Methods**

**BirA ligase**

Generating recombinant bioactive human HCS has proven difficult. Here, we used recombinant BirA ligase from *E. coli* as a surrogate for HCS to identify amino acid residues in histone H3 that are targets for biotinylation. Recombinant, purified BirA ligase was generously provided by John E. Cronan Jr., University of Illinois at Urbana-Champaign (20).

**Recombinant p67**

The polypeptide p67 comprising the 67 C-terminal amino acids from human propionyl-CoA carboxylase (GenBank accession #AAA60035), including the biotin-binding site (K669) (21), was cloned from Jurkat cell cDNA using forward primer 5′-GAATTCCTGCGTTCCCCGATG-3′ and reverse primer 5′-GGATCCTCATTCCAGCTCCACGAC-3′, and the vector pSTBlue (Novagen; San Diego, CA); the resulting plasmid was named “p67-pSTBlue.” p67-pSTBlue was sequenced verified at the University of Nebraska-Lincoln, DNA core facility. The insert encoding p67 was subcloned into vector pET30a (Novagen; San Diego, CA) by using EcoR1 and BamH1; this plasmid was named “p67-pET30a.” Rosetta 2 (DE3) cells were transformed with p67-pET30a and grown at 37°C to an optical density of 0.3 to 0.6 at 600 nm when IPTG was added for a final concentration of 1 mM. Incubation was continued for 16 h when cell pellets were collected by centrifugation at 2,700 g for 30 min. Pellets were resuspended in 10 mL of 50 mM Tris buffer, pH 7.5 and sonicating three times on ice at 30-sec intervals at maximal power (Aquasonic 250 T; VWR). Samples were centrifuged at 2,700 g for 30 min to remove cell debris. The supernatant was collected and protein was purified using His Trap FF columns on a AKTA protein purification system, according to manufacturer supplied protocols (Amersham; Piscataway, NJ). Aliquots of chromatographic fractions were separated on 18% polyacrylamide gels, and stained with Coomassie blue to identify fractions containing p67. Subsequent studies revealed that recombinant p67 contained covalently bound biotin (see Results), as judged by gel electrophoresis and streptavidin blotting as described previously.
The biotinylated fraction of p67 was removed by using avidin beads. Briefly, 10 mL of recombinant p67 from the His Trap FF column eluate was chromatographed over 3 mL of immobilized avidin (Pierce; Rockford, IL), and non-biotinylated p67 in the column flow-through was collected and quantified by measuring the absorbance at 280 nm.

**Biotinylation of p67, histones, and histone-based peptides by BirA ligase**

Previously, we developed a procedure to identify amino acids that are targets for biotinylation in histones using synthetic peptides and human serum as a source of BTD (17). Both amino acid substitutions and incorporation of modified amino acids (e.g., phosphorylation of serine) in the peptide chain were used to investigate their effects on biotinylation of adjacent lysine residues (16,17). Here, an analogous procedure was used to identify lysine residues in histones that are targets for biotinylation by BirA ligase.

The following substrates were tested as targets for biotinylation by BirA ligase: (i) biotin-free p67 was prepared as described above; (ii) bulk extracts of histones were prepared from human lymphoid (Jurkat) cell nuclei using 1 M HCl (22); (iii) commercial calf-thymus histone H1 was purchased from Calbiochem (La Jolla, CA); and (iv) synthetic peptides were produced based on the amino acid sequence in human histone H3 and quantified as described earlier (16). Biotinylation assays were conducted as follows: 0.75 μg of p67, 50 μg of commercial histone H1, 0.5 μg or 0.8 μg of synthetic peptides (see below) were incubated with 350 μg of BirA ligase in 100 μL of buffer containing 50 mM Tris acetate (pH 7.5), 0.2 mM biotin, 0.2 mM DTT, 5 mM ATP, and 100 mM MgCl₂. Samples were incubated at room temperature or 37°C for up to 180 minutes, depending on the substrate. The reaction was stopped by adding Tris-Glycine or Tricine loading buffer (Invitrogen) and heating at 95°C for 10 min. Following enzymatic biotinylation, histones were resolved using 18% Tris-Glycine, and p67 and peptides were resolved using 16% Tricine gels (17). Histones, p67, and proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with streptavidin-peroxidase (16,17,22).

**Results**

Identity of recombinant p67 was confirmed by sequencing of plasmid “p67-pET30a” and by molecular weight analysis following gel electrophoresis (Fig. 1A, lane 1). Purified p67 in eluates from the His-Trap FF columns exhibited a strong biotinylation signal (Fig. 1A, lane 2). We attributed biotinylation of recombinant p67 to the activity of endogenous BirA ligase in *E. coli*. In subsequent experiments, p67 was chromatographed over immobilized avidin which yielded a highly pure, essentially non-biotinylated fraction of recombinant p67 (Fig.1A, compare lanes 2 and 4). Note, however, that only <10% of p67 in the starting material was biotinylated, based on the observation that similar amounts of p67 were detected by staining with Coomassie blue before and after avidin column (Fig. 1A, compare lanes 1 and 3). When biotin-free p67 was incubated with recombinant BirA ligase for up to 2 minutes at room temperature, we observed a time-dependent increase in biotinylation of p67 (Fig. 1B). These data are consistent with the notion that both recombinant BirA ligase and its substrate p67 were biologically active. Having confirmed the activity and specificity of recombinant Bir A ligase, we studied its ability to biotinylate intact histones and short peptides based on histone amino acid sequences.

Biotinylation of histone H1 from calf thymus increased in a time-dependent fashion when incubated with BirA ligase and cofactors for up to 180 minutes (Fig. 2A). Histone extracts from Jurkat cells (containing histones H1, H2A, H2B, H3 and H4) were biotinylated by BirA ligase in a time-dependent fashion (Fig. 2B). Equal loading of lanes was confirmed by staining with Coomassie blue (Fig. 2C). This observation suggests that prokaryotic BirA ligase is capable of biotinylating all five major classes of human histones.
Finally, we used recombinant BirA ligase as a model to identify amino acid residues in human histones that are potential targets for biotinylation by HCS. The first peptides incubated with BirA ligase were denoted N$_{1-25}$ (spanning amino acids 1-25 in human histone H3) and N$_{15-39}$ (spanning amino acids 15-39). The strength of the biotinylation signals in peptides N$_{1-25}$ and N$_{15-39}$ was comparable (Fig. 3A, lanes 1 and 2), suggesting that the N-terminus in histone H3 contained targets for biotinylation by BirA ligase. Next, we incubated BirA ligase with peptide C$_{116-136}$ (spanning amino acids 116 through 136 in the C-terminus from histone H3); C$_{116-136}$ contains a lysine residue in position 122. No biotinylation was detected (Fig. 3A, lane 3), consistent with previous observations (16); C$_{116-136}$ was subsequently used as a negative control.

The next series of peptides incubated with biotin and BirA ligase focused on shorter sequences of amino acids from the N-terminus to identify potential biotinylation sites and understand the effects of neighboring amino acid residues on the strength of biotinylation. Short peptides were synthesized based on the sequence of the 25 N-terminal amino acids of human histone H3 as described earlier (16). Peptide N$_{1-9}$ included amino acids 1-9 of the N-terminal tail (ARTKQTARK); biotinylation of N$_{1-9}$ was detectable only after 2 h of incubation with BirA ligase (Fig. 3B), but the strength of the signal at 2 h was sufficiently high to suggest that K4 and K9 might be targets for biotinylation by BirA ligase. Peptide N$_{9-16}$ included amino acids 9-16 of histone H3 (KSTGGKAP); this peptide was a relatively poor target for biotinylation by BirA ligase (Fig. 3C) compared with peptide N$_{1-9}$ (Fig. 3B). Peptide N$_{16-23}$ included amino acids 16-23 of histone H3 (PRQLATK) and was a good target for biotinylation by BirA ligase; biotinylation was detectable after 30 minutes of incubation with BirA ligase (Fig. 3D), suggesting that K18 and K23 are potential targets for biotinylation by BirA ligase. Peptide N$_{18-25}$ also included K18 and K23 but was a weaker target for biotinylation by BirA ligase (Fig. 3E) than N$_{16-23}$ (Fig. 3D). The differences in biotinylation between peptides N$_{16-23}$ and N$_{18-25}$ could be explained by effects of neighboring amino acids which apparently interfered with Bir A ligase catalyzed biotinylation of peptide-lysine residues.

When both K4 and K9 in the N-terminal 9 amino acids were replaced with alanine, (K4,9A$_{1-9}$, negative control) no biotinylation was detectable (Fig. 4A, lane 1). Peptides containing only K9 or only K4 were biotinylated efficiently by BirA ligase (Fig. 4A, lane 2 and 3), indicating that both lysines were good targets for biotinylation.

Next, we focused on amino acids 9-16 in the N-terminus of H3. If both K9 and K14 in the N-terminal amino acids were replaced with alanine (K9,14A$_{9-16}$), no biotinylation signal was detected (Fig. 4A, lane 4). If only K14 was replaced with alanine (K14A$_{9-16}$), a strong biotinylation signal was detected (Fig. 4A, lane 5), suggesting that K9 was a good target for biotinylation by BirA ligase. If only K9 was replaced with alanine (K9A$_{9-16}$), only a weak biotinylation signal was detected (Fig. 4A, compare lanes 5 and 6), suggesting K14 was a moderate target for biotinylation.

Similar experiments focused on K18 and K23 of histone H3 showed that when both K18 and K23 were replaced with alanine (K18, 23A$_{18-23}$), there was no detectable biotinylation signal (Fig. 4A, lane 7). If only K23 was replaced with alanine (K23A$_{23}$), a weak biotinylation signal was detected (Fig. 4A, lane 8), suggesting K18 was a moderate target for biotinylation by BirA ligase. If only K18 was replaced with alanine (K18A$_{16-23}$), a strong biotinylation signal was detected (Fig. 4A, lane 9), suggesting that K23 was also a good target for biotinylation by BirA ligase. Likewise, if both K18 and K23 were replaced with alanine in peptide K18,23A$_{18-25}$, no biotinylation signal was detected (Fig. 4A, lane 10). If K23 was replaced with alanine (K23A$_{18-25}$), a weak biotinylation signal was detected (Fig. 4A, compare lanes 11 and 8). If K18 was replaced with alanine (K18A$_{18-25}$) a weak biotinylation signal was detected (Fig. 4A, lane 12). These results are different when compared to the biotinylation of peptide.
and suggests that amino acids 16 and 17, and 24 and 25 might affect biotinylation of K18 and K23 by BirA ligase.

Previous studies have suggested that some modifications of amino acids may affect the biotinylation of adjacent lysines. For example, phosphorylation of S10 in histone H3 caused a decrease in biotinylation of K9 (16). Here, we investigated whether modifications of amino acids affect biotinylation of neighboring lysine residues by BirA ligase, consistent with cross-talk among histone marks. For proof of principle, we focused on modifications and sequence variations in amino acids surrounding the biotinylation target K9.

A peptide spanning amino acids 6-13 in the N-terminus (N<sub>6-13</sub>) was a good target for biotinylation (Fig. 4B, lane 1). If K9 was replaced with alanine (K9A<sub>6-18</sub>), biotinylation signal was at about the level of background noise (Fig. 4B, lane 2; negative control). This is consistent with our observations described above that K9 is a target for biotinylation. Previous studies had suggested that R8 interferes with biotinylation of K9 by BTD (16). In contrast, R-to-A substitution in position 8 (R8A<sub>6-13</sub>) only slightly improved biotinylation of K9 by BirA ligase (Fig. 4B, compare lanes 2 and 3). Substitution of ornithine for R8 maintained the positive charge at position 8 (R8O<sub>6-13</sub>), and resulted in a strong biotinylation signal (Fig. 4B, lane 4). Our observations that charge interactions affect histone biotinylation by BirA ligase was further corroborated by the following finding. If a negative charge was introduced in position 10 by serine phosphorylation (S10S(p)<sub>6-13</sub>), biotinylation decreased to background levels (Fig. 4B, lane 5). Likewise, if the positively charged R8 was moved away from K9 biotinylation decreased compared with the native sequence (Fig. 4B, compare lanes 1 and 6).

Discussion

The covalent binding of biotin to histones in humans is thought to be mediated by BTD and HCS (6,14). We had previously used BTD to identify K4, K9, and K18 as targets for biotinylation in human histone H3. More recently, it was suggested that HCS is more important for biotinylation of histones (6,18). Consequently, we re-visited our previous studies of enzymatic biotinylation of histones by using recombinant BirA ligase as a surrogate for human HCS. In these studies we made a series of novel observations. First, we demonstrated that the polypeptide p67, which is frequently used to confirm biological activity of BirA ligase and HCS, is biotinylated by endogenous BirA ligase in <i>E. coli</i>. Hence, it is crucial to purify p67 by avidin chromatography before its use in biotinylation assays. Second, BirA ligase is a promiscuous enzyme which is capable of biotinylating human histones, lending further support to the notion that HCS plays an important role in histone biotinylation. We are currently pursuing the generation of recombinant, bioactive HCS in order to provide additional support regarding roles of HCS in chromatin biology. Third, K23 was identified as a novel biotinylation site by BirA ligase, in addition to confirming K4, K9, and K18 as targets for biotinylation (16). Fourth, biotinylation of lysine residues is subject to crosstalk among neighboring amino acids. For example, biotinylation of K9 is prevented by the phosphorylation of S10, a modification known to play a role in mitotic and meiotic chromosome condensation (23). Another example of crosstalk is not through histone modifications per se, but by charge patches in chromatin. For example, removal of positive charges or introduction of negative charges in the vicinity of K9 is associated with a decrease in the biotinylation of K9.

Evidence has been provided that streptavidin might bind to non-biotinylated histones (24). This is not a concern in the data presented here. First, if histones were probed with streptavidin prior to incubation with BirA ligase, no meaningful signal was detectable (Fig. 2B), consistent with previous studies in biotin-depleted histones (16,17).
We cannot formally exclude the possibility that HCS behaves differently than BirA ligase in regard to biotinylation of histones. However, we have already detected three of the biotinylation targets reported here (K4, K9, and K18) in eukaryotic cells by using site-specific antibodies (16). We have further demonstrated that knockdown of HCS in Drosophila and human lymphoid cells is associated with a substantial decrease in histone biotinylation (15,18). Importantly, biotinylated histone H3 is the most abundant class of biotinylated histones in Drosophila, and knockdown of HCS in flies is associated with phenotypes such as decreased life span and stress resistance (19).

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Abbreviations

BTD, biotinidase; HCS, holocarboxylase synthetase; K12BioH4, Biotinylated K12 on histone H4; SA, streptavidin.

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Fig. 1.
(A) Recombinant p67 undergoes biotinylation by endogenous BirA ligase. Abbreviations: CB = coomassie blue; SB = streptavidin blotting. (B) Time course of biotinylation of recombinant p67 by recombinant BirA ligase. Times denote the seconds of incubation of BirA ligase and p67 with biotin and cofactors. Note that all lanes shown were from the same blot, but that the order of lanes has been changed electronically to facilitate comparisons.
Fig. 2.
(A) Time course of biotinylation of histone H1 from calf-thymus by recombinant BirA ligase. (B) Biotinylation of human histones by BirA ligase. Histones were extracted from Jurkat cells and incubated with BirA ligase, biotin, and cofactors for the indicated times. Histones were resolved by gel electrophoresis and biotin in transblots was probed with streptavidin. (C) Equal loading of lanes was confirmed by staining with coomassie blue. Minutes of incubation of BirA ligase and histones H1 with biotin and cofactors are depicted. Note that all lanes shown were from the same blot, but that the order of lanes has been changed electronically to facilitate comparisons.
Fig. 3.
(A) The N-terminal tail in human histone H3 rather than the C-terminus is the primary target for biotinylation by BirA ligase. Synthetic peptides spanning amino acids 1-25 (lane 1), 15-39 (lane 2), and 116-136 (lane 3) in histone H3 were incubated with BirA ligase, biotin, and cofactors for 1 h. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase. (B-E) Biotinylation targets of BirA ligase in the N-terminal tail of human histone H3. Note that all lanes shown were from the same blot, but that the order of lanes has been changed electronically to facilitate comparisons.
Fig. 4.
(A) Biotinylation of K4, K9, K18, and K23 in the N-terminal tail in histone H3. Lanes 1-12 depict synthetic peptides based on overlapping sequences from the first 25 amino acids of histone H3. These peptides were incubated with BirA ligase, biotin, and cofactors. Peptides were resolved by gel electrophoresis and probed with streptavidin peroxidase. (B) Amino acid modifications and substitutions affect biotinylation of adjacent K9 in histone H3 by BirA ligase. Peptides based on amino acids 6-13, which includes K9 in histone H3 were incubated with BirA ligase, biotin, and cofactors for 2 h. Note that all lanes shown were from the same blot, but that the order of lanes has been changed electronically to facilitate comparisons.