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SCIENCE DIRECT
Journal of Nutritional Biochemistry 16 (2005) 416–418
Journal of Nutritional Biochemistry

Targeted and proximity-dependent promiscuous protein biotinylation by a mutant *Escherichia coli* biotin protein ligase☆

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Received 28 March 2005; received in revised form 28 March 2005; accepted 28 March 2005

Abstract

A method for general protein biotinylation by enzymatic means has been developed. A mutant form (R118G) of the biotin protein ligase (BirA) of *Escherichia coli* is used and biotinylation is thought to proceed by chemical acylation of protein lysine side chains by biotinoyl-5′-AMP released from the mutant protein. Bovine serum albumin, chloramphenicol acetyltransferase, immunoglobulin chains and RNAse A as well as a large number of *E. coli* proteins have been biotinylated. The biotinylation reaction is proximity dependent in that the extent of biotinylation is much greater when the ligase is coupled to the acceptor protein than when the acceptor is free in solution. This is presumably due to rapid hydrolysis of the acylation agent, biotinoyl-5′-AMP. Therefore, when the mutant ligase is attached to one partner involved in a protein–protein interaction, it can be used to specifically tag the other partner with biotin, thereby permitting facile detection and recovery of the proteins by existing avidin/streptavidin technology.
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Protein–protein interactions play important roles in virtually all cell processes. Enzymatic biotinylation seems to be an ideal tag for several reasons: (a) the biotin carboxyl forms amide linkages with the ε-amino groups of lysine residues; (b) a universal and highly conserved enzyme, biotin protein ligase (BPL), is known to catalyze formation of such linkages; (c) in all cells, naturally biotinylated protein species are rare such that background is very low; (d) the specific and extremely tight binding of biotin (K_D 10^{-13} to 10^{-15} M) to streptavidin and avidin allow very sensitive detection of biotinylated proteins by a wide variety of robust protocols; and (5) low-affinity forms of streptavidin and avidin allow efficient purification of biotinylated proteins under mild conditions. BPL is a universal and highly conserved enzyme that forms amide linkages with the ε-amino groups of lysine residues [1]. BPLs have exceptional acceptor protein specificities and thus are not general protein modification enzymes. Therefore, in order to use a BPL as a general biotinylating enzyme, the extraordinary specificity must somehow be overcome. A possible means to convert a BPL to a promiscuous protein biotinylation enzyme would be to mutate the protein to produce an enzyme that releases the normally enzyme-bound intermediate [1], bio-5′-AMP, from the active site. Bio-5′-AMP is a mixed anhydride and therefore should act as a nonspecific chemical protein biotinylation reagent. Moreover, the instability of acyladenylates such as bio-5′-AMP should result in proximity-dependent biotinylation because any acyladenylate molecule that diffuses far from the enzyme should be inefficient in protein acylation due to low concentration and the high rate of acyladenylate hydrolysis. In our work [2], we examined three mutant proteins of the *Escherichia coli* BPL (BirA) characterized by Kwon and Beckett [3] and Xu and Beckett [4] for their ability to nonspecifically biotinylate *E. coli* proteins

☆ This paper was presented at the international symposium "Vitamins as Regulators of Genetic Expression: Biotin as a Model" NAFTA Satellite Meeting to the XXV National Congress of Biochemistry held on December 3–4, 2004, in Ixtapa, Zihuatanejo, Mexico. This meeting was sponsored by Sociedad Mexicana de Bioquimica A.C.; Programa de Doctorado en Ciencias Biomedicas, Universidad Nacional Autonoma de Mexico; Laboratorios Roche–Syntex, Mexico; and Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico.

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doi:10.1016/j.jnutbio.2005.03.017

in vivo. One of these, R118G, acted as a promiscuous and proximity-dependent biotinylation reagent. Indeed, the R118G protein was found to efficiently biotinylate itself as well as a variety of proteins that normally lack this modification.

The wild-type and three mutant BirA proteins (R118G, G115S and Δ1–34) were expressed from a plasmid-based *tac* promoter. High-level expression of each protein was confirmed by SDS-PAGE. To assay for the ability to biotinylate proteins in a promiscuous manner, the extracted proteins of each strain were analyzed for biotin attachment by SDS-PAGE separation followed by Western blotting with a streptavidin–AP conjugate. The extracts of all four strains showed the expected biotinylated BCCP band plus a band that corresponded to biotinylated BirA (see below). However, many additional biotinylated protein bands were seen in the cell extract in which the R118G mutant protein had been expressed. In contrast, the wild-type and N-terminal deletion protein extracts showed very weak labeling of noncognate proteins with the mutant protein giving stronger signals. Surprisingly, the G115S protein showed no labeling of noncognate proteins. The labeled bands seen in the R118G extract were strong evidence for promiscuous protein biotinylation; thus, we proceeded to purify and study the mutant protein. To test in vitro biotinylation by R118G, C-terminal hexahistidine-tagged (His-tagged) wild-type and R118G BirA proteins were purified by Ni-NTA agarose chromatography using purified apo BCCP (a natural BirA substrate) as a specific substrate and commercial BSA and/or RNAse A as a promiscuous biotin acceptor. Western blotting with streptavidin–AP showed that both BSA and RNAse A became biotinylated, with R118G being much more active than the wild-type protein. In contrast, the specific substrate, apo BCCP, showed similarly efficient biotinylation by the two BirA proteins. We envision two possible mechanisms for promiscuous protein biotinylation. The first possibility is that lysine side chains of promiscuous acceptor proteins somehow gain access to the BirA active site due to the R118G mutation. The second possibility is that biotinylation of such proteins is not a direct enzymatic activity of the R118G protein but is due to chemical acylation by bio-5′-AMP released from the mutant active site. We strongly favor the second possibility because BSA trapped in a dialysis bag was slowly biotinylated in an ATP-dependent manner when R118G, biotin and ATP were placed outside the bag (which excluded molecules greater than 10 kDa). The time courses of R118G-dependent biotinylation of apo BCCP and RNAse A were determined. Biotinylation of the promiscuous acceptor protein increased throughout the 24-h incubation whereas biotinylation of apo BCCP reached a maximal level within 30 min. Thus, biotinylation of a promiscuous acceptor was much slower than biotinylation of the specific substrate, a result consistent with chemical acylation by released bio-5′-AMP.

The crude extract of the bacterial strain that expressed high levels of the R118G protein showed many biotinylated protein bands. One of the stronger bands had the molecular weight of BirA, suggesting that R118G had become biotinylated. A less intense band was found at the same migration position for the other enzymes. We therefore tested the possibility that the purified wild-type and R118G proteins had become biotinylated in vivo and found that only the R118G protein was highly modified. When R118G was expressed in an *E. coli* biotin auxotroph in the presence of ^{14}C-labeled biotin, the molar ratio of covalently attached biotin to BirA was 0.4. Therefore, appreciable protein modification occurred during expression under biotin-sufficient conditions.

In order to obtain unbiotinylated R118G for further in vitro characterization of the self-biotinylation reaction, we expressed the protein in a strain defective in biotin biosynthesis and added avidin just prior to induction to deplete the growth medium of biotin avidin. The lack of biotin available for the self-biotinylation resulted in preparations of unmodified proteins. These preparations were shown to self-biotinylate in vitro. The extent of R118G BirA self-biotinylation in vitro was measured by assay of the attachment of ^{14}C-biotin. When incubated with ^{14}C-biotin and ATP for 24 h, the molar ratio of covalently attached biotin to BirA was 0.2.

As discussed above, biotinylation of promiscuous acceptor proteins by bio-5′-AMP released by R118G would be expected to modify acceptors in a proximity-dependent manner. This follows from the rapid hydrolysis of bio-5′-AMP (a mixed anhydride) when in free solution at elevated pH that is expected to limit its ability to acylate distant proteins. Our first indication of proximity-dependent biotinylation was the finding that a 10-fold molar excess of BSA failed to completely suppress self-biotinylation of R118G, suggesting that the released bio-5′-AMP preferentially modified R118G rather than the promiscuous acceptor. For a more general test system, we used the His-tag present on the R118G C-terminus. In this case, the biotin acceptors were the heavy and light chains of an antibody that recognizes a penta-histidine tag. We compared the relative biotinylation rates of the antibody when it was either bound to the R118G C-terminus or free in solution while bound to a His-tagged derivative of CAT (the antibody was blocked with a His-tagged CAT protein prior to addition of the His-tagged R118G). Hence, in the first case, the acceptor protein would be close to the BirA active site, whereas in the second case, the acceptor protein would be randomly distributed in the solution. In some experiments, we added BSA or RNAse A as a promiscuous competitor protein. The heavy and light chains of the antibody were found to be good biotinylation acceptors when bound to the R118G C-terminus but were significantly less modified when the antibody was first blocked with His-tagged CAT to prevent binding of the antibody to the BirA His-tag. This blocking step reduced biotinylation

of the antibody heavy chain to 22% of that seen in the absence of His-tagged CAT (the intensity of signal for light chain in the presence of His-tagged CAT was too weak for quantitation). The decrease in antibody biotinylation upon blocking cannot be attributed to competition for biotinylation between the His-tagged CAT protein and the antibody since addition of BSA or RNAse A (both of which are much better biotin acceptors than CAT) or CAT lacking a His-tag had little or no effect on biotinylation of the R118G-bound antibody. Details of this and the other experiments are given in Ref. [2].

References

[1] Chapman-Smith A, Cronan JE. The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. Trends Biochem Sci 1999;24:359–63.

[2] Choi-Rhee E, Schulman H, Cronan JE. Promiscuous protein biotinylation by *Escherichia coli* biotin protein ligase. Protein Sci 2004; 13:3043–50.

[3] Kwon K, Beckett D. Function of a conserved sequence motif in biotin holoenzyme synthetases. Protein Sci 2000;9:1530–9.

[4] Xu Y, Beckett D. Evidence for interdomain interaction in the *Escherichia coli* repressor of biotin biosynthesis from studies of an N-terminal domain deletion mutant. Biochemistry 1996;35:1783–92.