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Previews

Biotin Synthase: Enzyme or Reactant?

Biotin synthase catalyzes formation of a thiophane ring through a radical mechanism that is difficult to reconstitute in vitro. Choi-Rhee and Cronan [1] measure a turnover of 20–60 equivalents of biotin in vivo, but also find that turnover renders the protein susceptible to proteolytic destruction.

How much biotin does a bacterium need to survive? Is it possible that biotin synthase is technically not an enzyme but rather a catalytic reactant that is evolved to accomplish only one turnover? These are questions that are often raised in discussions of the mechanism of biotin synthase [2], an iron-sulfur protein that, while undeniably chemically powerful, is perhaps nature's slowest enzyme.

Biotin is an essential enzyme cofactor that is used to catalyze transcarboxylation reactions, including crucial reactions in fatty acid biosynthesis, gluconeogenesis, and branched-chain amino acid catabolism. Many bacteria, including *Escherichia coli*, use biotin only for the enzyme acetyl CoA carboxylase, the first step in fatty acid biosynthesis, and require a minimum of ~100–200 molecules per cell. Although other enzymes in the *E. coli* biotin biosynthetic pathway [3] are well-characterized and undergo steady-state turnover with maximal in vitro turnover numbers of ~1 min^{-1}, biotin synthase has proven obstinate and resists efforts to reconstitute steady-state turnover in a defined assay of purified enzyme. Numerous authors have reported the in vitro production of less than one equivalent of biotin per biotin synthase monomer, but there is little consensus on the reasons for such low activity.

Choi-Rhee and Cronan design an elegant series of experiments that address whether biotin synthase is capable of multiple turnovers in *E. coli* [1]. This in vivo measurement is in principle difficult since the endogenous expression level of biotin synthase is very low and because biotin is split between pools of free and protein bound cofactor. The first issue is overcome by overexpressing his-tagged biotin synthase (His_6-BioB) under control of an arabinose-inducible promoter. The second issue is overcome by massively overexpressing, under control of an IPTG-inducible T7 promoter, biotin ligase (BirA) and a truncated, his-tagged form of the acetyl CoA carboxylase biotinylated subunit (His_6-`AccB) that can accept biotin but does not form an active enzyme complex. They then use a combination of anti-pentahistidine antibodies, (^{35}S)-methionine labeling, and streptavidin to quantify the levels of each protein and of total biotinylated protein separated by denaturing and nondenaturing gel electrophoresis. Following expression of the respective proteins, further protein synthesis was halted with tetracycline and enzyme turnover was initiated by addition of dethiobiotin (DTB) to the media.

The measurement of biotin production is straightforward. The levels of both His_6-BioB and biotinylated His_6-`AccB can be quantified on the same gel using either Western blots or (^{35}S)-methionine incorporation, and the ratio gives 20–60 equivalents of biotin produced per initial biotin synthase monomer. Although a true kinetic analysis is premature, 20 turnovers observed in 4 hr gives a minimum turnover number of ~0.08 min^{-1}, essentially equivalent to the maximal rate of ~0.07 min^{-1} for one turnover observed in vitro [4]. However, the in vivo measurement is complicated by the unexpected finding that enzyme turnover renders the enzyme susceptible to proteolytic degradation.

In carefully controlled experiments, Choi-Rhee and Cronan find that addition of DTB to the culture medium results in a 50%–90% depletion of the level of His_6-BioB after 16 hr incubation. This depletion is not observed in the absence of DTB or in the presence of biotin. The complete disappearance of the His_6-BioB band following turnover, with no obvious degradation products, could be due to partial unfolding of the protein and subsequent proteolysis of at least the his-tagged N- and C termini, but more likely of the entire protein. In vitro, we have observed that the C terminus of BioB is susceptible to limited proteolysis during purification (~4–8 kDa fragments removed), but the N terminus is highly resistant to cleavage (unpublished data). Thus it would seem that the observation of complete degradation is most consistent with conversion to a proteolytically susceptible unfolded state following turnover.

Biotin synthase contains two iron-sulfur clusters (Figure 1). A $[4Fe\text{-}4S]^{2+}$ cluster interacts with S-adenosylmethionine [5] and is essential for the generation of a 5′-deoxyadenosyl radical [6] and the abstraction of hydrogen atoms from DTB [7]. In addition, a $[2Fe\text{-}2S]^{2+}$ cluster is found in the core of the protein ~4.7 Å from DTB [8] and is likely involved in attaching sulfur to the substrate. Alternate mechanisms have been proposed for the mechanism of sulfur insertion into DTB that differ in the role proposed for the $[2Fe\text{-}2S]^{2+}$ cluster [2]. One mechanism proposes that a sulfide from the cluster is attached in a stepwise manner to the C9 and C6 positions of DTB, with concomitant reduction and loss of the residual cluster [4]. An alternate mechanism suggests that reduction and loss of the cluster precedes catalysis [9], and that sulfur insertion is from an enzyme bound cysteine persulfide that forms either during cluster degradation or via the action of an exogenous cysteine desulfurase [10]. Both mechanisms maintain that loss of the $[2Fe\text{-}2S]^{2+}$ cluster is required, either as a result of or a precondition for turnover. However, the mechanisms differ in how they rationalize the inability to carry out multiple turnovers in vitro.

In the cluster insertion mechanism, the regeneration of the $[2Fe\text{-}2S]^{2+}$ cluster is essential to regenerate active enzyme. Marquet and coworkers attempted the in vitro reconstitution of the $[2Fe\text{-}2S]^{2+}$ cluster following turnover and looked for resurrection of the enzyme, but observed only minimal activity [11]. Johnson and co-

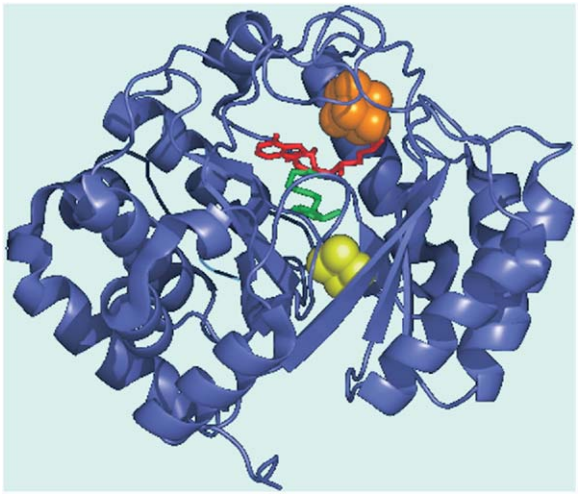

Figure 1. Ribbon Depiction of the Biotin Synthase Monomer

The protein backbone forms an $(\alpha\beta)_8$ barrel around the $[4Fe\text{-}4S]^{2+}$ cluster (orange), *S*-adenosylmethionine (red), DTB (green), and the $[2Fe\text{-}2S]^{2+}$ cluster (yellow) [8]. The C terminus is disordered and extends 31 residues from bottom center.

workers noted that the in vitro reconstituted $[2Fe\text{-}2S]^{2+}$ cluster is not vibrationally equivalent to the native cluster [12], suggesting minor differences in the surrounding protein fold or environment that could explain why in vitro cluster reconstitution does not correlate with activity. In vivo cluster assembly is likely carried out through the concerted action of several proteins from the iron-sulfur cluster (ISC) assembly system [13]. In the persulfide insertion mechanism, the inability to sustain multiple turnovers of biotin production is explained as due to severe product inhibition by biotin [10]. Under the conditions employed by Choi-Rhee and Cronan, this inhibition is presumably relieved by coupling of biotin to His_6-`AccB as catalyzed by BirA, and turnover would be limited only by the rate of biotin dissociation. It should be noted that biotin inhibition has not been observed by other groups working with biotin synthase.

Regardless of the mechanism, turnover of biotin synthase is clearly accompanied by increased degradation of the protein. This could be due to partial unfolding of the protein in the absence of the $[2Fe\text{-}2S]^{2+}$ cluster. A unique and unexpected feature of biotin synthase is the incorporation of an arginine guanidino group, as well as three cysteine thiolates, as ligands to this cluster [8]. In the absence of metal coordination, the arginine would likely become protonated and be repelled from the hydrophobic interior of the protein, providing a plausible role for this conserved residue in sensing the presence of the cluster. Unfolding of the protein may be an evolved feature that facilitates repair of the otherwise deeply buried $[2Fe\text{-}2S]^{2+}$ cluster. In the absence of efficient cluster repair, BioB degradation may facilitate more rapid downregulation of biotin production after only a few turnovers, a feature that could conserve metabolic energy and promote stationary-phase survival under nutrient-deprived conditions.

Joseph T. Jarrett
Department of Biochemistry and Biophysics
University of Pennsylvania
Philadelphia, Pennsylvania 19104

Selected Reading

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