Minireview

Structural enzymology of biotin biosynthesis

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Abstract Over the last years, significant progress has been made in the understanding of the genetics and enzymology of the biosynthetic pathway of the vitamin biotin. The enzymes catalyzing the last four steps of this pathway, from pimeloyl-CoA to biotin, provide an ensemble of intriguing reaction mechanisms, which are presently being unravelled. The three-dimensional structures for three of these enzymes are known and provide a framework to which on-going mechanistic studies can be related.

Key words: Enzyme mechanism; Dethiobiotin synthetase; Biotin synthase; Aminotransferase

1. Introduction

Biotin (vitamin H) is a cofactor in a number of enzymatic carboxylation reactions, and is present only in very small amounts in mammalian cells. The biosynthesis pathways of this essential cofactor have been studied in detail in *Escherichia coli* [1,2], *Bacillus sphaericus* [3], *Bacillus subtilis* [4], *Saccharomyces cerevisiae* [5] and higher plants [6]. Because biotin biosynthesis is unique to plants and many microorganisms, enzymes of this pathway are potential targets for the development of safe antimicrobial drugs and herbicides [7].

The biotin biosynthesis genes in *E. coli* are located in a cluster, which consists of five genes, *bio*ABFCD [2]. With the exception of *bio*C, which might be involved in an early step of biotin biosynthesis, these genes code for enzymes catalyzing the four last steps in the pathway (Fig. 1). In *E. coli*, the *bio* operon is negatively regulated by the action of BirA, the repressor of biotin biosynthesis [8] which binds to the translational control region of the *bio* operon [9]. In *B. subtilis* a single *bio* operon was found, *bio*WAFDBI [4], whereas in *B. sphaericus* two gene clusters, *bio*XWF and *bio*DAYB, were described [3]. In addition to these studies, whole genome sequencing efforts have provided information on biotin biosynthetic genes from a number of other microorganisms. From these studies it appears that the last four steps of biotin biosynthesis, from pimeloyl-CoA to biotin, are common to most bacteria and plants (Fig. 1). The different chemical events necessary for the formation of biotin from the precursor pimeloyl-CoA provide an ensemble of intriguing enzymatic reaction mechanisms, which have attracted considerable attention during the last decade.

2. 7-Keto-8-amino pelargonic acid synthase

7-Keto-8-amino pelargonic acid (KAPA) synthase (also known as 8-amino-7-oxononanoate synthase, EC 2.3.1.47) is a homodimeric, pyridoxal phosphate-dependent enzyme that catalyzes the first committed step of the pathway (Fig. 1). The enzyme subunit has a molecular mass of about 42 kDa. Amino acid sequence comparisons and the 3D structure of the enzyme [10] suggested that KAPA synthase belongs to the fold type I family of vitamin B_6-dependent enzymes and is a member of the aminotransferase subclass II [11]. Within this subclass, KAPA synthase forms a small subfamily of α-oxoamine synthases, with 5-aminolevulinate synthase, serine palmitoyltransferase, and 2-amino-3-oxobutyrate CoA ligase as the other members [10,12]. Indeed, studies using recombinant KAPA synthase from *B. sphaericus* demonstrate that the reaction mechanism employed by KAPA synthase is very similar to that of 5-aminolevulinate synthase, thus providing evidence that the similarities extend beyond structure and also include the chemistry [12]. This work led to the design of KAPA synthase inhibitors, with the most potent inhibitor 8-amino-7-oxo-8-phosphononanoic acid (K_i 7 μM) derived from the structure of a putative reaction intermediate [13].

The crystal structure of KAPA synthase (Fig. 1) from *E. coli* was determined in the apo- and holoform [10] and as the enzyme–product external aldimine complex [14]. The subunit of KAPA synthase folds into two domains, a large domain containing a seven-stranded β-sheet, and a smaller domain, comprising the C-terminal part of the polypeptide chain. The C-terminal consists of a four-stranded β-sheet with helices on one side. The N-terminal part of the polypeptide chain forms a small three-stranded β-sheet, which extends the sheet of the C-terminal domain. Pyridoxal phosphate is bound in a crevice between the two subunits in the dimer and residues from both subunits are involved in cofactor binding. In the holoenzyme, the cofactor is covalently linked to the ε-amino group of a lysine residue, a residue conserved in the whole enzyme family. The X-ray analysis of the holoenzyme and the enzyme–product external aldimine complex provided the structural framework for a mechanistic proposal for catalysis by KAPA synthase which may be of general importance for other members of the α-oxoamine family [14]. Essential steps

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Abbreviations: DAPA, 7,8-diaminopelargonic acid; KAPA, 7-keto-8-aminopelargonic acid; PLP, pyridoxal 5′-phosphate; DTBS, dethiobiotin synthetase

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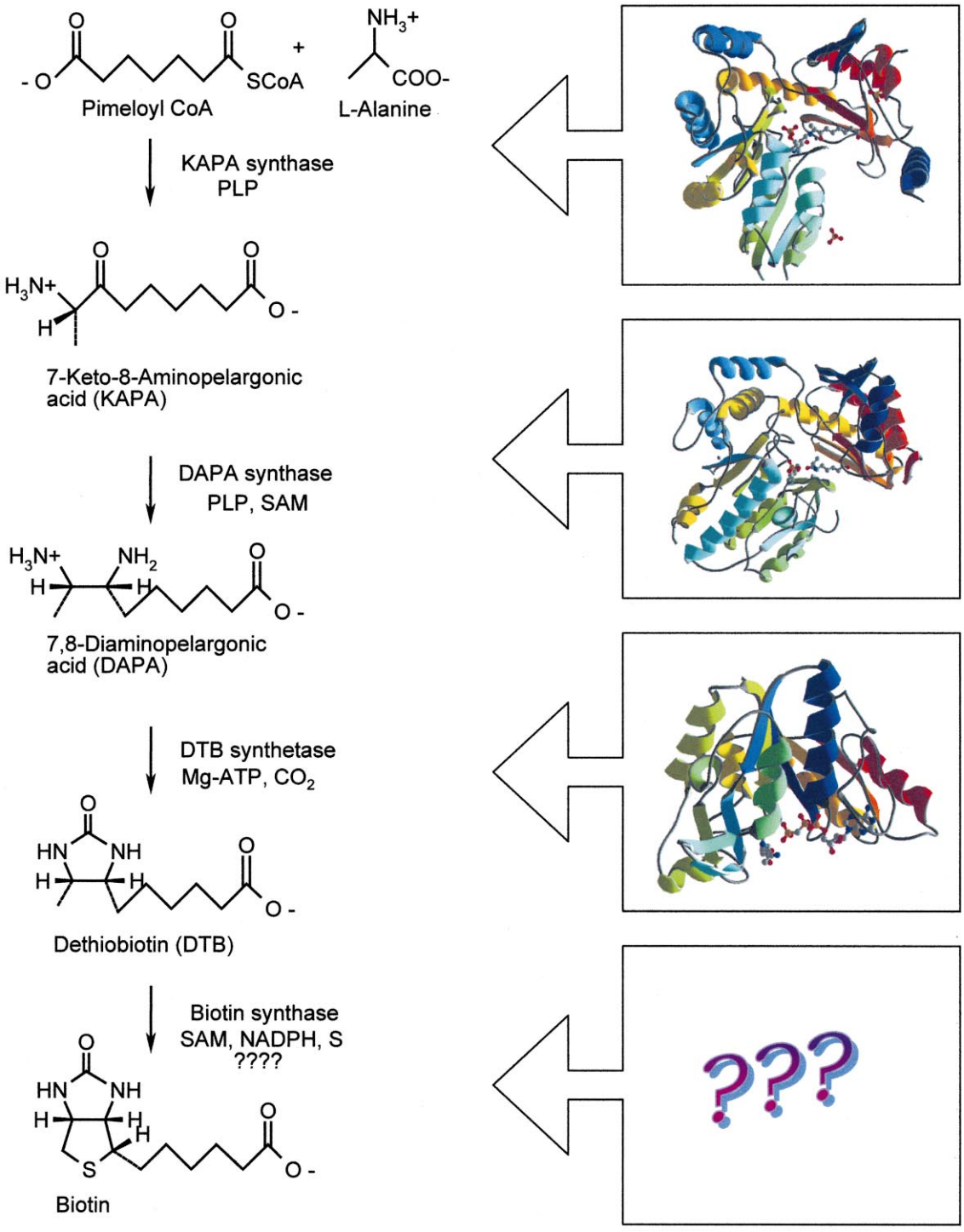

Fig. 1. The pathway of biotin biosynthesis, common for most microorganisms and plants. Schematic views of the 3D structure of the enzymes are included if known.

of this mechanism [12,14] are formation of an external aldimine between pyridoxal 5′-phosphate (PLP) and the substrate, L-alanine. Abstraction of the C2-H proton of the aldimine, possibly by Lys236 (*E. coli* numbering), leads to a quinonoid intermediate, which then attacks the thioester carbonyl of pimeloyl-CoA. Release of HSCoA produces a β-ketoacid aldimine, which after decarboxylation is converted into the product.

3. 7,8-Diaminopelargonic acid synthase

The antepenultimate step in this pathway, the conversion of KAPA to 7,8-diaminopelargonic acid (DAPA), is catalyzed by DAPA synthase, another vitamin B_6-dependent enzyme (Fig. 1) [15]. DAPA synthase from *E. coli* is a homodimer with a molecular mass of 94 kDa [15,16] and contains 429 residues per monomer [2]. The enzyme uses *S*-adenosyl-L-methionine

as amino group donor, an unusual feature among aminotransferases [15]. Sequence comparisons suggested that DAPA synthase belongs to a subclass of the fold type I family of PLP-dependent enzymes [17] and the crystal structure analysis [18] identified DAPA synthase as a member of the aminotransferase subclass II of this fold family [11].

The overall structure of DAPA synthase is very similar to the structure of KAPA synthase. Together with the amino acid sequence homology, albeit weak, this suggests that the two enzymes might be evolutionarily related, and possibly derived from a common ancestor [2]. From the structure of the non-productive complex of the holoenzyme with the substrate KAPA, the location of the active site and residues involved in substrate binding were identified.

An intriguing observation is the absence of transamination in DAPA synthase catalysis [19]. Since KAPA is an amino acid, it could in principle act as an amino group donor, and not only as an amino group acceptor in the reaction. The structure of the enzyme–PLP–KAPA complex [18] provided the structural explanation for how the enzyme avoids this unwanted side reaction.

4. Dethiobiotin synthetase

This enzyme catalyzes the ATP-dependent formation of dethiobiotin from DAPA and CO_2. Dethiobiotin synthetase (DTBS) is a homodimer, with a subunit molecular mass of 24 kDa. The crystal structure of the apo-enzyme [20–22] revealed that the polypeptide chain folds into a single domain, containing a seven-stranded parallel β-sheet, with helices on both sides. This fold was also observed in other phosphotransferases, such as adenylate kinase and p21ras. In the homodimer, the two equivalent active sites, about 25 Å apart, are located at the interface between the subunits. Crystallographic studies of complexes of DTBS with nucleotides, substrates and substrate analogs [23–26] provide detailed insights into the active site and conformational changes upon binding of substrate, and revealed conserved amino acids involved in binding of substrates and catalysis.

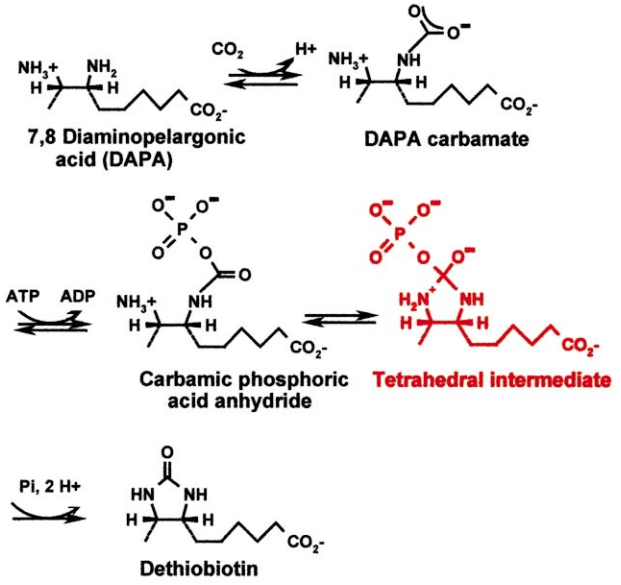

Fig. 2. Reaction mechanism for DTBS. The intermediate which has not yet been verified by crystallographic studies is shown in red.

The catalytic steps during the turnover of DAPA and CO_2 to dethiobiotin were already proposed by Eisenberg and coworkers [27] (Fig. 2). The first step of the reaction is the formation of a DAPA carbamate. The regiospecificity of DAPA carbamylation was addressed by two independent crystallographic studies which both showed specific carbamate formation at N7 of the DAPA molecule [23,24]. These studies also suggest that carbamylation of DAPA is part of the enzyme-catalyzed reaction cycle. Independent evidence for carbamate formation at this position was provided by the use of ^{1}H and ^{13}C NMR pulse-chase experiments, and synthesis and inhibition patterns of carbamate analogs [28].

A second reaction intermediate is formed by transfer of the γ-phosphoryl group from ATP to a carbamate oxygen [29,30]. Compelling evidence for the existence of this intermediate, a mixed carbamic–phosphoric acid anhydride, in the catalytic cycle of DTBS comes from time-resolved crystallographic studies [25]. The phosphoryl transfer step shows inversion of the configuration at the phosphorus atom, consistent with an in-line attack by the carbamate oxygen onto the phosphorus atom of ATP. A key feature in the structure of the enzyme–intermediate complex determined at 1.6 Å resolution is two magnesium ions, bridging the phosphates at the cleavage site. These magnesium ions compensate the negative charges at both phosphate groups after phosphoryl transfer and contribute to the stabilization of the reaction intermediate [25]. Another important interaction of the phosphate oxygens of ATP is a salt bridge to the invariant residue Lys15. The analysis of site-directed mutants at this position of DTBS is consistent with key roles of Lys15 in γ-phosphoryl transfer to the carbamate and stabilization of the phosphorylated intermediates, and binding of ATP [31].

The final step of DTBS catalysis involves nucleophilic attack of the N8 nitrogen onto the carbonyl oxygen of the carbamate, with subsequent release of the phosphate group and formation of the ureido ring of dethiobiotin (Fig. 2). It was postulated that a third reaction intermediate is formed in this step [23,31]. However, this intermediate has so far been elusive and there is as yet little evidence for its existence in the catalytic cycle. Observed differences in the binding of substrate, DAPA and the product dethiobiotin at the active site of the enzyme suggest considerable displacements of substrate atoms during the ring closure step of the catalytic reaction, best described as rotation of the ureido ring of dethiobiotin by almost 180° [26].

Only minor conformational changes of the enzyme are observed in the various enzyme complexes. A significant exception involves the phosphate binding loop (Gly8-X-X-X-X-X-Gly14-Lys15-Thr16) which changes conformation upon binding of ADP, ATP and ATP analogs [23]. This displacement brings main chain atoms of residues 12, 13 and 14 close to the phosphate groups of the nucleotide resulting in the formation of hydrogen bonds. Replacement of one of the residues in this loop, Thr11Val, results in a 24 000-fold increase in the K_M for ATP, with little change in k_{cat}, thus emphasizing the importance of this loop in the binding of the cosubstrate, ATP [31].

5. Biotin synthase

The final step of biotin biosynthesis is chemically the most challenging: the insertion of a sulfur atom between the non-

reactive methyl and methylene carbon atoms adjacent to the ureido ring of dethiobiotin (Fig. 1). In enzymological terms, this reaction of the pathway is also least understood. Compelling evidence was assembled that the product of the *bio*B gene, in the literature referred to as biotin synthase, is essential for this reaction to occur [32–36]. However, there is at present no in vitro assay where biotin synthase sustains biotin formation at catalytic rates.

The *bio*B gene of *E. coli* codes for a polypeptide of about 38 kDa [2,32] and the active species of the protein appears to be the homodimer. When isolated aerobically in the oxidized form, the monomer contains a [2Fe–2S] cluster [32,37,38], upon reduction, however, a [4Fe–4S] cluster is formed [37–39]. Incubation with excess iron and sulfide results in a protein containing two [4Fe–4S] clusters per dimer, which might be the redox-active species in the anaerobic enzyme [38,39].

Biotin synthase contains a sequence motif encompassing three cysteine residues, the cysteine box, which is also found in other *S*-adenosylmethionine-dependent enzymes containing Fe–S clusters such as anaerobic ribonucleotide reductase and pyruvate formate lyase activating enzyme. The results of mutagenesis studies focusing on these three conserved cysteine residues (Cys53, Cys57, and Cys60) in *E. coli* biotin synthase [40,41] are consistent with these residues contributing ligands to the irons of the cluster.

The conversion of dethiobiotin to biotin by biotin synthase requires the presence of a reducing system, usually NADPH, flavodoxin (or ferredoxin) and flavodoxin (or ferredoxin) NADP+reductase, and *S*-adenosylmethionine. The latter does not act as the sulfur donor in the reaction, but is cleaved to methionine and a 5′-deoxyadenosyl radical [36], possibly facilitated by the Fe–S clusters [37]. This radical can then react further with the substrate, by abstraction of a hydrogen atom at the methyl and a methylene group of the substrate. This requires two equivalents of *S*-adenosylmethionine per molecule of biotin formed, consistent with the observed stoichiometry [35,36].

The origin of the sulfur incorporated in biotin has been the focus of a number of studies. Recent work provides evidence that the sulfur is donated by biotin synthase itself [42,43], in fact by the Fe–S cluster [42]. It appears that biotin synthase acts as a substrate rather than an enzyme in this reaction, which explains the failure to sustain multiple turnovers. In order to restore activity the Fe–S centers have to be regenerated. In a recent study [44], it was shown that the NifS protein from *Azotobacter vinelandii*, the C-DES protein from *Synechocystis* as well as rhodanese from bovine liver all can catalyze incorporation of sulfur derived from cysteine into apobiotin synthase, resulting in active reconstituted biotin synthase. Importantly, however, these proteins had no effect on the turnover of biotin synthase in the in vitro assay. The question then arises whether the present failure to experimentally demonstrate multiple turnover is due to missing components in the assay mixture or indeed, if the last step of biotin synthesis is enzyme-catalyzed at all. The latter implies that each 'biotin synthase' molecule functions only once, making biotin biosynthesis a very costly enterprise [43].

There are thus a number of intriguing mechanistic questions concerning the enzymology of this last step in biotin biosynthesis, which are as yet unresolved. A major obstacle is the absence of structural information on biotin synthase and it can be expected that the determination of the 3D structure will advance our understanding of the function of this elusive enzyme.

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