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Review Commentary

In vivo formation of C—S bonds in biotin. An example of radical chemistry under reducing conditions

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ABSTRACT: The last step in the biosynthesis of biotin involves the formation of carbon–sulfur bonds at non-activated carbons catalyzed by biotin synthase. *S*-Adenosylmethionine (AdoMet) and an electron source are essential for activity in cell-free systems. This important finding connects biotin synthase to a family of enzymes, namely pyruvate–formate lyase, anaerobic ribonucleotide reductase and lysine 2,3-aminomutase, which use the same cofactors. Additional experimental data led to the proposition of the following general mechanism. The carbons to be functionalized are first activated by homolytic cleavage of the C—H bonds, initiated by the deoxyadenosyl radical produced by a monoelectronic reductive cleavage of AdoMet with NADPH as electron source. The electron transfer system involves flavoproteins and very likely the [Fe—S] center of biotin synthase. NADPH and the flavoproteins can be replaced by photoreduced deazaflavin. By using a deuterated substrate, a deuterium transfer into deoxyadenosine has been observed, indicating that biotin synthase should be closely related to lysine 2,3-aminomutase, which uses AdoMet as a surrogate of vitamin B12. The source of sulfur, the nature of the immediate sulfur donor and hence the mechanism of trapping of the intermediate radicals are still unknown. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: biotin; *in vivo* C—S bond formation; radical chemistry; reducing conditions

INTRODUCTION

A few important sulfur-containing molecules, e.g. penicillin, biotin and lipoic acid, involve the formation of C—S bonds at non-activated carbons in the last step of their biosynthesis (Scheme 1).

These reactions, although chemically related, follow very different pathways and illustrate well the diversity of solutions that nature has elaborated to achieve the same chemistry. The reaction catalyzed by isopenicillin N synthase requires oxygen and the homolytic cleavage of the valine β C—H bond is performed by an iron–oxo species,[1,2] a process which is now well documented in many metalloenzymes catalyzing oxidation reactions. We shall show that a completely different mechanism is operative in the other two cases. This mechanism is far from being completely elucidated, but significant progress has recently been made.

HISTORICAL BACKGROUND

The understanding of the last step in biotin biosynthesis represents an extremely difficult problem. Although it has been tackled a long time ago, conclusive data have been very scarce,[3] mainly because of the absence of activity in cell-free systems, preventing *in vitro* enzymology.

Our group was able, however, to obtain significant results through *in vivo* experiments with intact cells (*Escherichia coli* or *Bacillus sphaericus*). We could exclude the two reasonable hypotheses, based on known enzymatic pathways, which could account for the activation of the carbons to be functionalized, namely the involvement of hydroxylated[4] or unsaturated[5,6] intermediates.

We could also show that the primary thioderivative **1** (X=H) was converted into biotin by *E. coli* or *B. sphaericus*[7] (Scheme 2).

This led us to postulate that **1** (X=H or X≠H) was a very likely intermediate and that it was probably formed by a direct sulfur insertion since hydroxylation had been excluded.[7] The reaction occurs with racemization,[5] as shown by stereochemical studies carried out with dethiobiotin bearing a chiral methyl group.[8] This result is consistent with a radical mechanism. It could, of course, also be compatible with an intermediate carbenium ion, as considered for the cytochrome P450 hydroxylations,[9] but even in that case this process does not appear as a very general one.

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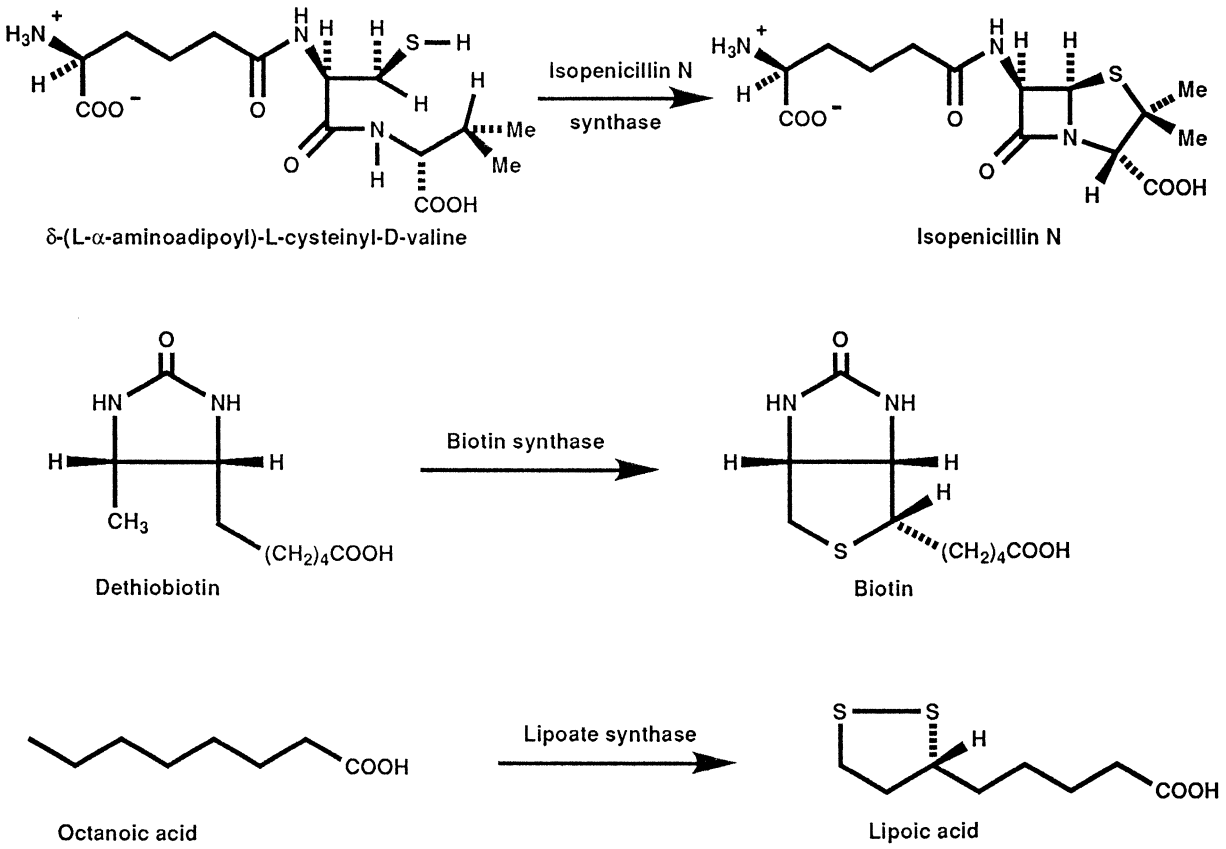


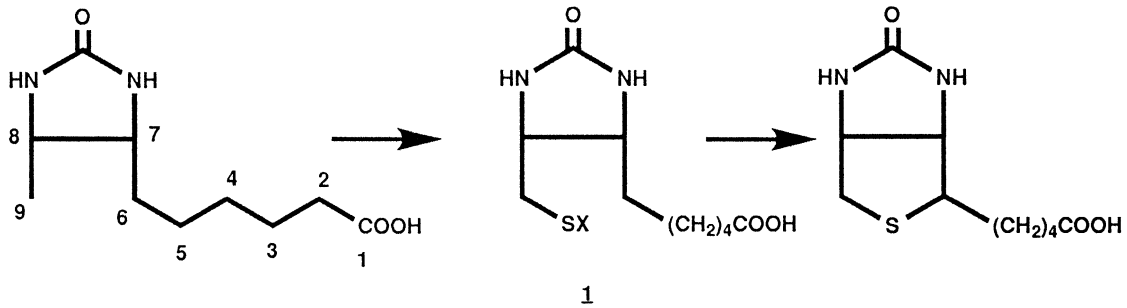
Scheme 1

Similar conclusions were reached in parallel studies of lipoic acid biosynthesis in *E. coli*:[3,10] there is no loss of hydrogens except those which are replaced by sulfur; 6- and 8-hydroxyoctanoic acid are not substrates whereas 6- and 8-mercaptooctanoic acid are transformed into lipoic acid. The reaction occurs also with racemization[11] at the methyl group. The hypothesis that the same mechanism is involved for the two molecules is also strongly supported by the sequence homologies of biotin synthases and lipoate synthases.[12,13]

There are many conserved residues, in particular, as shown in Scheme 3 a cysteine triad and a cluster Y(F)NHN, which certainly belong to the active site. Interestingly, these very homologous sequences have no similarity with the sequence of isopenicillin N synthase[14,15] or of any other known protein.

RECENT DEVELOPMENTS

After many reports of unsuccessful attempts to obtain activity in acellular systems, a positive result was announced by Ifuku *et al.*[16] in 1992. By adding different potential sulfur donors to the cell-free extracts of an *E. coli* strain overproducing biotin synthase, they found that *S*-adenosylmethionine (AdoMet) was very efficient in improving the activity. This very important result opened up a new area.



Scheme 2

BIOTIN SYNTHASE

A. thaliana	KTGG**C**SED**C**SY**C**PQSSRYST	79	aa	AGLTA**YNHN**LDTS-
S. cerevisiae	KSGG**C**SED**C**KY**C**AQSSRNDT	80	aa	AGLTA**YNHN**IDTS-
E. coli	KTGA**C**PED**C**KY**C**PQTSRYKT	77	aa	AGLDY**YNHN**LDTS-
S. marcescens	KTGA**C**PED**C**KY**C**PQSPRYKT	77	aa	AGLDY**YNHN**LDTS-
B. sphaericus	KSGY**C**PED**C**GY**C**SQSSKSTA	78	aa	AGVDR**YNHN**LNTS-
B. flavum	KTGG**C**PED**C**HF**C**SQSGLFES	76	aa	AGVHR**YNHN**LETA-
M. leprae	KTGG**C**PED**C**HF**C**SQSGLFMS	76	aa	IGVHR**YNHN**LETA-

LIPOATE SYNTHASE

E. coli	LGAI**C**TRR**C**PF**C**DVAHGRPV	77	aa	TPPDV**FNHN**LENV-
H. influenzae	LGAI**C**TRR**C**PF**C**DVAHGKPL	76	aa	NPPDV**FNHN**LENV-
S. cerevisae	LGDT**C**TRG**C**RF**C**SVKTNRTP	73	aa	CGLDV**YAHN**LETV-

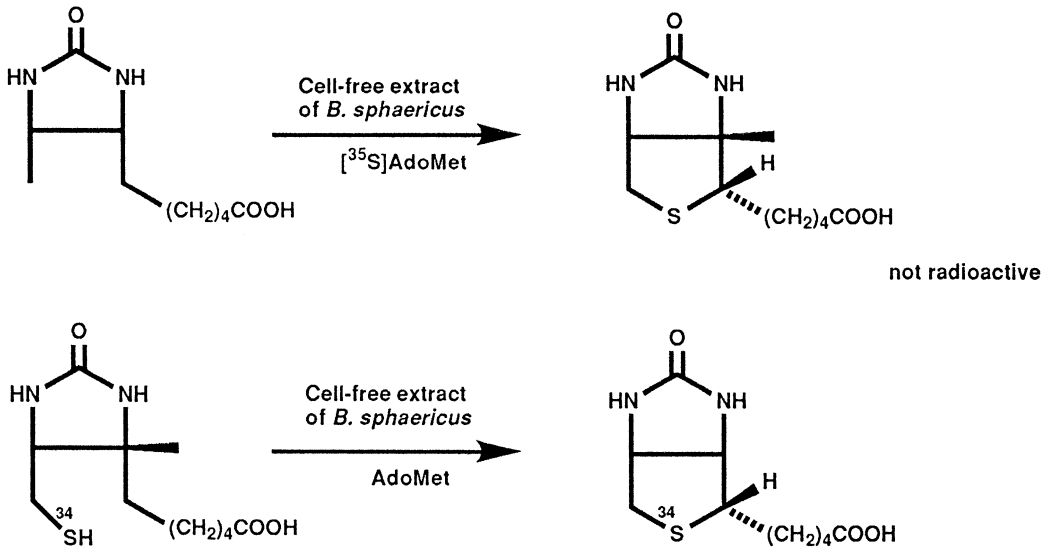
Scheme 3

Some time later, we showed that AdoMet was not the sulfur donor:[17] the sulfur of [^{35}S] AdoMet was not incorporated into biotin and the ^{34}S-labeled thiol which also requires AdoMet for cyclization was transformed into [^{34}S]biotin (Scheme 4).

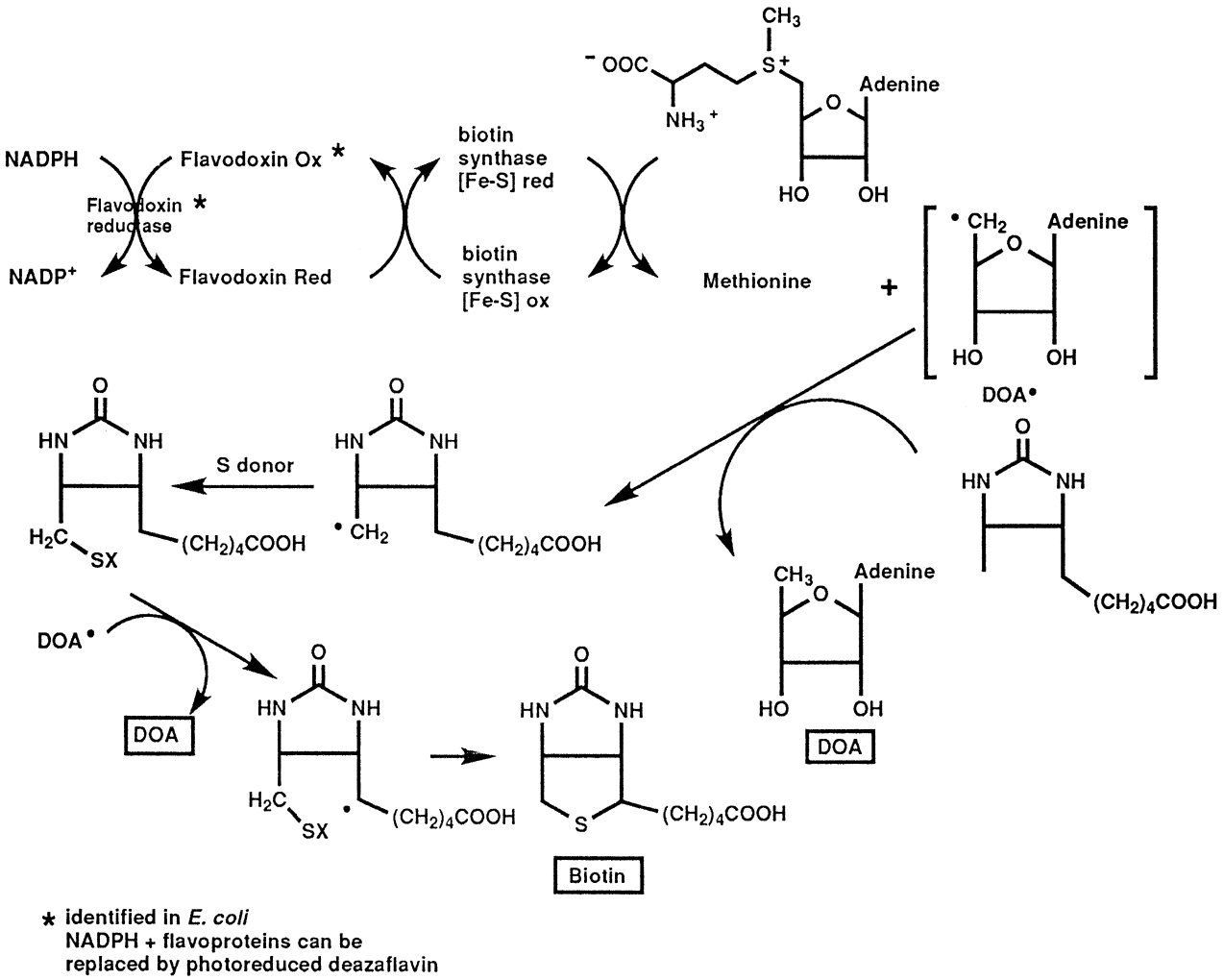
This led us to propose[17] that biotin synthase could belong to the fascinating class of enzymes which use AdoMet as a source of deoxyadenosyl radical, namely:

- pyruvate–formate lyase (PFL), which converts pyruvate into acetyl CoA and formate;[18,19]
- anaerobic ribonucleotide reductase (ARR), which transforms nucleotides into deoxynucleotides;[20]
- lysine 2,3-aminomutase (LAM), which catalyses the isomerization of α- and β-lysine.[21]

The three enzymes are involved in anaerobic metabolism and generate a deoxyadenosyl radical (DOA$^{\bullet}$) by reduction of AdoMet. The exact mechanism of this reduction is still unknown and the deoxyadenosyl radical has to be stabilized by some group of the enzyme, maybe a metal ion or the [Fe—S] cluster[22].



Scheme 4



Scheme 5

It has been now established that PFL and ARR are radical proteins, a glycyl radical being generated by abstraction of a glycyl hydrogen probably by the deoxyadenosyl radical. In LAM, on the other hand, the deoxyadenosyl radical removes $H^{\bullet}$ directly from the substrate. Although they catalyze very different reactions, the first event in the mechanism of the three enzymes is the homolytic cleavage of a C—H bond. As pointed out above, such a homolytic C—H cleavage is also the postulated first event in biotin synthase mechanism.

We have therefore proposed the mechanism depicted in Scheme 5 assuming that the methyl group is functionalized first, since only the primary thio derivative **1** (Scheme 2) was converted into biotin.[7]

Although not completely understood, the first part of the pathway is now supported by many experimental data. The final events, namely the nature of the sulfur donor and the mechanism of the C—S bond formation, remain very mysterious.

Characterization of biotin synthase and of its electron-transfer system

We are working with biotin synthase from two organisms, *B. sphaericus* and *E. coli*. The enzyme of *E. coli* is also being studied by several groups at Shiseido,[16] DuPont[23,24] and Lonza.[25]

Some consistent results have been obtained by different teams. Both enzymes have been purified from recombinant strains. They are dimers (2×37–38 kDa).[26,27] Biotin synthase is an [Fe—S] protein. This was first described for the *E. coli* enzyme.[23] The iron and sulfur content and the UV–visible spectrum are compatible with one [2Fe—2S] cluster per monomer. In the oxidized form [2Fe—2S] centers are silent in EPR. After reduction by dithionite, an EPR signal, characteristic of a [2Fe—2S] center, is observed, but with a very weak intensity, indicating that the reduced species is not stable.

We have shown that the UV–visible spectra of the enzymes of *B. sphaericus* and *E. coli* are very similar.[26]

When the *B. sphaericus* enzyme was reduced with dithionite, the EPR signal, different from the corresponding one in *E. coli*, could not be interpreted.[28] The iron–sulfur center is apparently still more unstable in this case.

With purified biotin synthase, NADPH is essential for activity.[23,25] In *E. coli*, two electron-transfer proteins, flavodoxin and flavodoxin reductase[25,29] have been identified. An *in vitro* system capable of transforming dethiobiotin into biotin[24] thus contains *E. coli* biotin synthase, flavodoxin, flavodoxin reductase, AdoMet, NADPH, Fe^{2+}, DTT and some other cofactors, the role of which has not been clearly rationalized. In our hands, however, this system is not catalytic and the amount of biotin produced is sub-stoichiometric with respect to the enzyme; routinely, 0.5 nmol of biotin is formed from 1 nmol of biotin synthase.

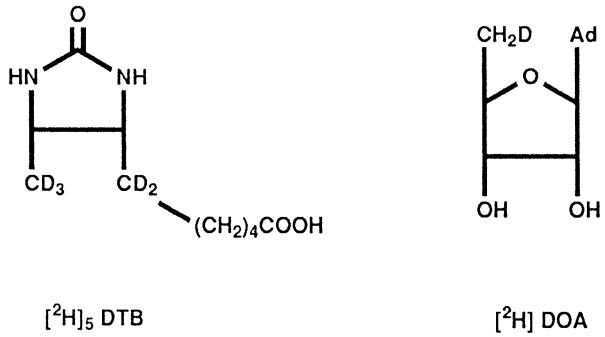
The corresponding flavoproteins of *B. sphaericus* have not yet been identified. An artificial electron donor, namely photoreduced deazaflavin (DAF),[30] has been shown to replace NADPH and the proteic electron-transfer system in PFL[31] and ARR.[32] We have observed that the same was true for biotin synthase of *B. sphaericus* (and also *E. coli*).[33] This led us to adopt another minimal *in vitro* assay called the DAF system, which contains biotin synthase, AdoMet and deazaflavin, in a DTT-containing Tris buffer. With this system, the yield of transformation of dethiobiotin into biotin is very low (1 nmol of enzyme gives 0.02 nmol of biotin), but the important result is that this transformation takes place and that no other protein is necessary, when electrons are supplied.

Experimental evidence for the role of AdoMet

The proposed hypothesis (Scheme 5) implies that AdoMet is cleaved into methionine and deoxyadenosine, which should therefore be produced in equimolecular amounts. This had to be checked. Another important problem to solve was to correlate the amount of AdoMet used in the reaction with the amount of biotin produced. In the three related enzymes already mentioned, the DOA$^\bullet$ acts as a catalyst. With PFL and ARR, it produces, directly or indirectly, an intermediate glycyl radical which is regenerated at each turnover. With LAM, DOA gives back one hydrogen to the substrate.[22] In the biotin case, the radical is trapped by a sulfur species and AdoMet should rather be considered as a co-substrate. The consumption of AdoMet is expected to be at least 1 mol per mole of biotin to produce the radical on the methyl group. If the DOA$^\bullet$ is used to remove H$^\bullet$ on the other carbon, a second mole of deoxyadenosine should be produced.

The quantification has been carried out using AdoMet labeled with ^{35}S or ^{3}H on the adenine moiety to determine methionine and deoxyadenosine, respectively. The deoxyadenosine/biotin or methionine/biotin ratio was 2.8 ± 0.2.[34] This value, above 2, indicates an abortive consumption of AdoMet and it is not possible to conclude if 1 or 2 mol are necessary for the production of 1 mol of biotin.

The next question to address is whether the DOA$^\bullet$ itself abstracts the substrate hydrogens, as in LAM, or if there is a relay of a protein radical. To answer this question, a pentadeuterated substrate, [$^2H_5$]DTB, was synthesized (Scheme 6).



[^{2}H]$_5$ DTB [^{2}H] DOA

Scheme 6

If there is a direct transfer, deoxyadenosine will incorporate ^2H whereas an intermediate protein radical would imply the formation of non-labeled deoxyadenosine, since the system is not catalytic. The mass spectrum of the recovered deoxyadenosine clearly shows deuterium incorporation.[28] This excludes the occurrence of a glycyl (or any other) radical on the protein and indicates that biotin synthase is more closely related to LAM than to PFL and ARR, as far as the C—H bond cleavage is concerned.

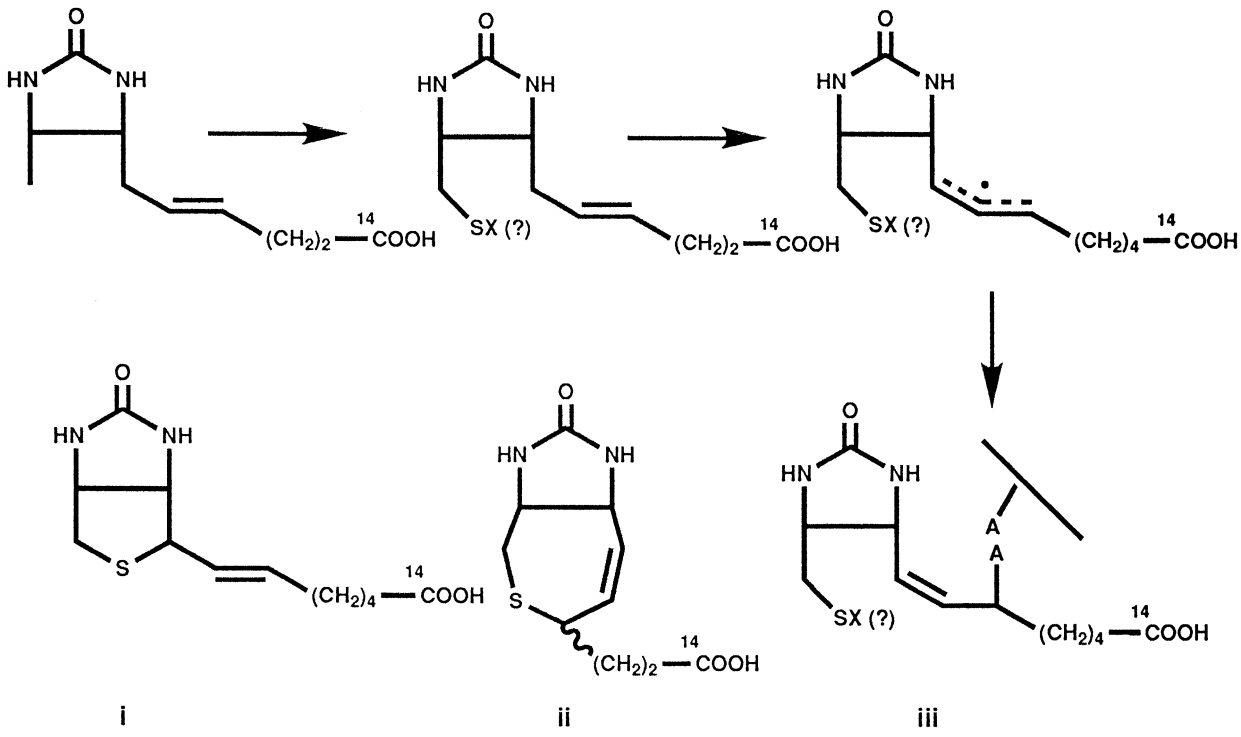
4,5-Dehydrodethiobiotin, the first suicide substrate of biotin synthase?

Enzyme-generated radicals have been described as being able to form covalent bonds with the protein, e.g. with Tyr,[35] His[36] or Cys[37] residues.

We have tested the behavior of 4,5-dehydrodethiobiotin, assuming that the intermediate allylic radical could react according to several pathways, either cyclization into dehydrobiotin (i) or with ring extension (ii) or addition to the protein (iii) (Scheme 7).

A 1- ^{14}C-labeled substrate was used in this study. The amount of transformation product was too low for identification,[38] but covalent binding to the protein was clearly established. A labeled band corresponding to biotin synthase is visible on the electrophoresis gel. The labeling is abolished in the absence of AdoMet or in the presence of an excess of cold dethiobiotin (Fig. 1).[38]

Hence we think that 4,5-dehydrodethiobiotin is the first suicide substrate of biotin synthase, although the kinetic arguments for a k_{cat} inhibition cannot be provided as long as the system is not catalytic.



Scheme 7

The sequencing of the labeled protein, which is under way, will enable us to characterize one (or a few) residue(s) of the active site.

The nature of the sulfur donor

This is still an unsolved problem. In our early experiments with a crude cell-free extract of *B. sphaericus*, we included [^{35}S]cysteine in the assay and we found that [^{35}S]biotin was produced but with a specific activity much lower than that of cysteine.[17] This result indicates that the immediate sulfur donor is not cysteine but is produced from cysteine through enzymes present in the cell free extract.

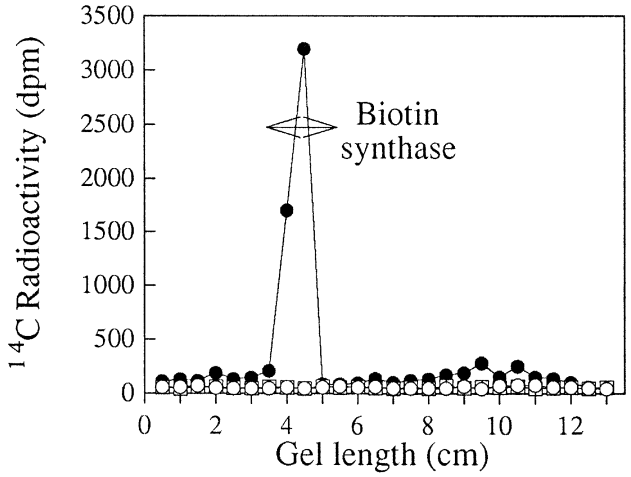


Figure 1. Radioactive electrophoresis gel profile of a crude cell-free extract of *B. sphaericus* incubated with [^{14}C]-(*E*)-4,5-dehtdrodethiobiotin in the presence of AdoMet and NADPH[38]. (●) complete assay; (○) without AdoMet; (□) with 20 equiv. DTB

Another interesting argument in connection with this discussion is the above-mentioned experiment with the DAF system, which reveals that biotin is produced from dethiobiotin without an external sulfur source except DTT present in the buffer. When [^{35}S]cysteine was added to this system, the biotin produced was not radioactive,[33] an expected result since the assay did not contain any other enzyme. Sulfur was introduced, however, suggesting that it is given by biotin synthase itself, either by the iron–sulfur cluster or another sulfur species covalently bound to the protein. Another hypothesis is that DTT could be the sulfur donor under these non-physiological conditions.

Experiments are in progress to establish which of these possibilities is correct. The elucidation of the nature of the sulfur donor is an obligatory step in understanding its insertion mechanism.

CONCLUSION

Biotin synthase represents a challenge for mechanistic enzymology. The very low efficiency of the *in vitro* system reveals that some other cofactor(s) are very likely

necessary for turnover. Nif S[39,40] and Nif U[41] gene products, which have been shown to be involved in the biosynthesis of [Fe–S] clusters, could be missing. The search for these unknown cofactors will be the next step of our investigation.

Among the many fascinating mechanistic problems raised by enzymes, those related to homolytic reactions are, with a few exceptions, the least understood. If significant progress has been made in the elucidation of the mechanisms of oxidation by oxygen-consuming metalloproteins, the family of enzymes which were discussed here, which use the reductive cleavage of AdoMet as a source of deoxyadenosyl radical, are only starting to be unraveled. They belong to anaerobic metabolism and have to use reducing conditions to generate radicals. Interestingly, biotin synthase is the first enzyme of the family found in aerobic bacteria. Indeed, these AdoMet-dependent enzymes are involved in different transformations which have in common only the first step, namely the homolytic cleavage of a C—H bond. It seems reasonable to assume that some other members of the family, catalyzing other reactions, could be discovered.

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