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ON THE MECHANISM OF ACTION OF GTP-TRANSFORMING ENZYMES

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Abstract: Mechanisms for the ring conversion of GTP into cofactor precursors by GTP cyclohydrolases and molybdopterin synthase are proposed and discussed in the context of the crystal structure of the GTP cyclohydrolase I - 2'-deoxy-GTP complex. The mechanisms suggest that common features of acid-base catalysis may underly the reactions catalysed in all three cases.

GTP-cyclohydrolases I and II (GTPCH-I and II) catalyse the first commited stages in the biosynthetic pathways leading respectively to folate and biopterin cofactors, and to flavin cofactors.[1] The involvement of this wide range of cofactors in metabolic transformations including the biosynthesis of nucleic acids, of essential amino acids and hormones, and in such fundamental processes as oxidation gives the GTP-cyclohydrolases special importance.[2] The occurence and genetics of these enzymes in many species has been widely studied but their mechanism of action has been little more than surmised from the overall chemical reactions that they catalyse. The availability of genes coding for these enzymes has made it possible to obtain significant quantities of the wild type enzymes and mutants[3]. Recently, a crystal structure of GTPCH-I at 3Å resolution has been described[4] providing the first substantial basis for a molecular understanding of the mechanism of action. Structural information is also available for GTPCH-II from two sources, *Bacillus subtilis* and *Escherichia coli*;[5] the enzymes from the two sources appear to have little sequence homology. A third cofactor class, the Mo cofactors (molybdopterin), also appears to be derived from GTP.[6]

GTPCH-I apparently catalyses a complex series of reactions in which the imidazole and ribose rings of GTP are opened, C-8 is lost as formate, and the ribose fragment undergoes Amadori rearrangement and recyclisation to form dihydroneopterin triphosphate (figure 1a).[7] Nothing is known about the elementary steps of these reactions. GTPCH-II catalyses part of this process leading simply to loss of C-8 as formate and may follow only one part of the GTPCH-I pathway (figure 1b).[8] Precursor Z has been proposed to be derived from GTP via a complex series of reactions that could be related to the cyclohydrolases although none of the carbon atoms of GTP appears to be lost in the transformation (figure 1c).[6]

The fact that these three enzymes each lead to cofactors of great biosynthetic significance suggests that they may be early enzymes in evolutionary terms as has been implied by Golding in the case of B_{12}- mediated rearrangements.[10] Further, since they supply cofactors, it is possible that they are not highly evolved with respect to catalytic efficiency because a small quantity of cofactor would suffice the demands of many metabolic enzymes of higher efficiency. Two of these enzymes, GTPCH-I and precursor Z synthase, catalyse a complex series of events including ring openings and recyclisations. In each case, the purine ring is converted into a pteridine.

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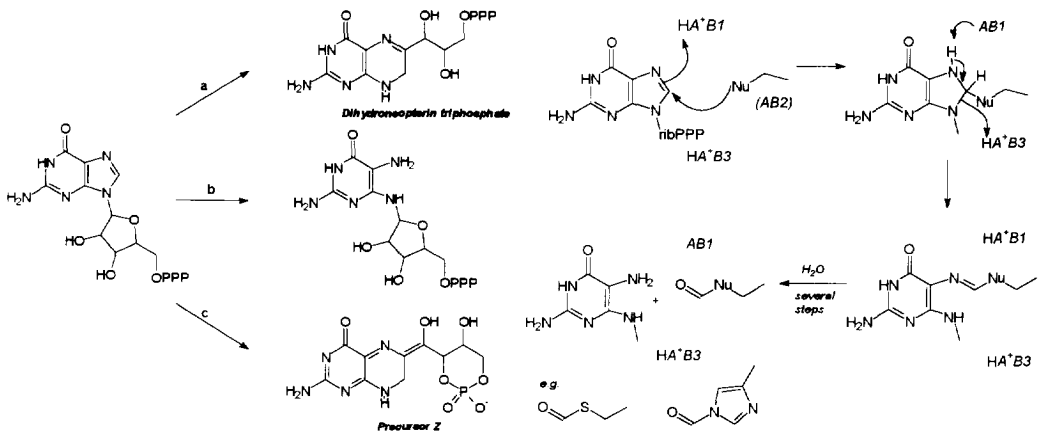


Figure 1. (left) Reactions catalysed by GTP-transforming enzymes. a: GTP cyclohydrolase I, b: GTP cyclohydrolase II, c: molybdopterin synthase. **Figure 2.** (right) Generalisation of a possible mechanism for ring opening catalysed by GTP-cyclohydrolase I or II

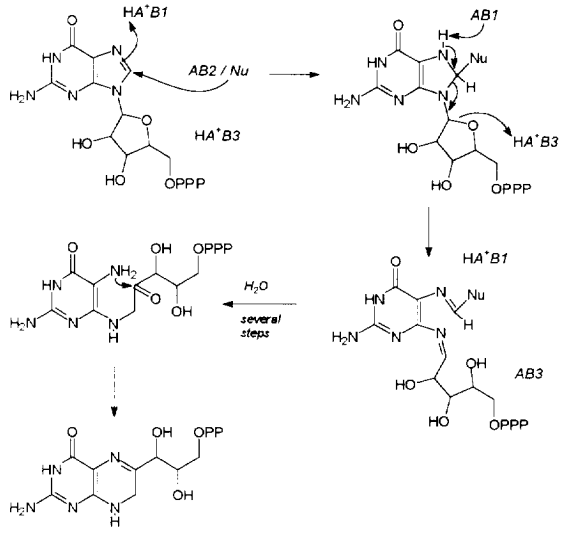
It is obvious that GTPCH-II operates by net hydrolytic cleavage of the imidazole ring of the purine system and it is most probable that the first events in the reactions catalysed by the other enzymes occur at this site too. Thus it is possible that there is a mechanistic relationship between the three enzymes at their active sites even if the homology in sequence and structural terms is low, a possibility consistent with the concept of the ancient evolutionary ancestry of these enzymes. We propose here reasonable mechanisms that account for the biosynthetic processes observed and that would be appropriate for the reactivity of amino acid side chains at an enzyme's active site. Where structural information exists, the proposals are evaluated in that context.

GTP-Cyclohydrolases I and II The reactions can be described most simply through the participation of three acid/base catalysts shown as AB1, AB2, and AB3 in figure 2; it is of course possible that two of the so-identified acid/base catalysts could be the same amino acid residue. In this analysis, the functions of these groups are respectively to donate a proton to N-7 thereby activating the C=N bond to nucleophilic addition (AB1), to activate a nucleophile to attack C-8 (AB2), and to protonate the leaving group N-9 (AB3). The identity of the nucleophile is important. If AB2, possibly as a glutamate or aspartate or unprotonated histidine, activates water to form a hydroxide as nucleophile, an intermediate N-5 formylpyrimidine would result. Similar activation of water by AB3, proton donation by AB2 to the formate oxygen and by AB1 to the 5-NH could catalyse the hydrolysis of the N-formylpyrimidine intermediate. Such an intermediate is normally proposed for GTPCH-1 (see below[7]) but it seems clear that an enzyme would not evolve to form such a stable intermediate as an amide without being able to catalyse its subsequent cleavage.

The question therefore arises as to the possibility that nucleophilic catalysis may occur in the cleavage of the purine. Such a reaction might be intiated by the unprotonated form of AB2 itself or by another amino acid residue, for example cysteine. In these ways, a much more reactive formyl intermediate is obtained, such that hydrolysis by water would require minimal catalytic intervention by the enzyme (figure 2). The above chemistry

is fully consistent with well established reactions in benzo-1,3-azole chemistry, including purines.[11]

The mechanism outlined so far is sufficient to describe the course of the reaction catalysed by GTP cyclohydrolase-II. The extension of this outline mechanism to GTPCH-I requires a small change in the site of action of AB3. If protonation occurs at the ring O of ribose, AB3 can be seen to activate concomitant opening of purine and furanose rings (figure 3); this possibility has also been recognised independently by Bacher[12,13] and is stereoelectronically reasonable in the conformation adopted by 2'-deoxy-GTP in the active site of the crystallised protein.[4] AB3 of a suitable pK_a can then be used as an acid-base catalyst for the Amadori rearrangement removing a proton from O-2' and subsequently donating it to the putative N-7. In such a sequence, significant movements of heavy atoms would be required so that the purine C-8 can be removed and a suitable geometry adopted for cyclisation to give the pteridine ring system. It is possible that the cyclisation following tautomerisation could take place after release of the keto intermediate from the active site. As with GTPCH-II, the question of the primary nucelophile that attacks C-8 of the purine arises.



The recently published crystal structure for GTPCH-I from *E.coli*[4] makes it possible to evaluate these mechanistic hypotheses in the context of an active site structure. In carrying out this evaluation it is important to realise that the active site is at a three way interface between subunits and domains of the enzyme. Hence it is reasonable to expect that the enzyme may undergo substantial conformational changes during the complex reaction course. Moreover, the correlation between chemical mechanism and protein structure is dependent upon the similarity between the productive complex and the position in which the 2'-deoxy-GTP present in the crystal binds. It is also impossible to rule out a mechanistic contribution by the 2'-hydroxyl group.

Figure 3. A possible chemical interpretation of the reaction catalysed by GTP-cyclohydrolase I.

With these caveats in mind, AB1 can be associated with His179 which presents an NH within 4Å of N7 of GTP. AB3 similarly can be assigned to His112 which appears to hydrogen bond with O' of the ribofuranose ring. The question of the primary nucleophile is, however, more complex. There is ample room for water activated by AB2 to serve; however, as with GTPCH-II, this would lead to a very stable intermediate, an N-5 formylpyrimidine. Many years ago, there was a report that this compound is transformed by GTPCH-I into product.[7] However, no intermediates were found to be released into solution by the enzyme and it is possible that the N-formylpyrimidine is an alternative substrate and not an intermediate. It has been proposed that the sulfur atoms of Cys110 or Cys181 might act as nucleophiles;[12,13] they are both within reasonable distance of C-8 although the crystal data suggest that the angle of attack on the imidazole ring would be far from optimal. In order to obtain the best nucleophiles, the disulphide bridge would require to be reduced, a reaction that would certainly increase

the flexibility for attack at the imidazole ring. However, no reducing cofactors have been described for the enzyme and it appears that the crystalline enzyme, in which the disulphide is undoubtedly present, is capable of transforming GTP.[4,13] There is precedent for the reaction of disulphides as nucleophiles in alkylation[14] and in this respect, they are very similar to thioethers. A few examples of the addition of thioethers to sp^2 carbon centres have been described[15] and it is therefore possible that a disulphide could behave similarly in GTPCH-I, with the geometric caveat mentioned above. There is, however, no precedent to our knowledge for such a reaction at an enzyme's active site. A third alternative nucelophile is a histidine residue. Through such a nucleophile, the formate would be retained at a higher level of activity leading to an acyl imidazole which, as noted above, would hydrolyse with minimal catalytic assistance to formate. Once again, however, this mechanism is not well precedented. The action of histidine as a nucleophile in enzymes is limited largely to phosphoryl transfer[16] although it is common in non-enzymic chemistry.[17] We conclude that the most probable mechanism of action of the GTP cyclohydrolases therefore involves general acid/base catalysis involving histidine residues close to the purine ring in GTP cyclohydrolase I. It is also possible that residues further from the site of chemical transformation could be involved through hydrogen bonding and it has indeed been suggested that the γ-phosphate of GTP plays such a role in GTP cyclohydrolase I[13] in partnership with Ser135 in the Amadori rearrangement phase of the reaction.

Molybdopterin synthase The possibility that the C-8 proton of GTP exchanges with solvent in the reaction catalysed by GTP cyclohydrolase has been suggested by Bacher.[19] This reaction can, however, be understood through the protonation of N-7 by AB1 as before and the deprotonation of C-8 by AB2 to form an ylide intermediate reminiscent of the behaviour of thiamine pyrophosphate. Indeed, the exchange of the C-8 proton and similar sites in heterocyclic compounds is well documented.[20] As will be described below, this observation suggests a possibility for the conversion of GTP into precursor Z in the biosynthesis of molybdopterin. The conversion of GTP into precursor Z is the most complex transformation of the three. Until recently, there has been no evidence to indicate the origin of precursor Z. Rajagopalan has reported that labelled GTP is incorporated at low levels into precursor Z and has suggested a reaction scheme to account for the observed incorporation. A specific observation was that C-8 of the purine appeared to be incorporated into C-1' of the side chain of precursor Z.[9] The mechanism proposed by Rajagopalan requires the *insertion* of C-8 between C-2' and C-3' of the ribose ring. Such an outcome involves the breaking of four covalent bonds in an energetically uneconomical manner. On the other hand, it is possible to interpret the conversion of GTP into precursor Z using the three acid/base catalysts described for the GTPCH-ases and we suggest two possible mechanisms.

If AB1 protonates N-7 as before and AB2 generates the transient ylide by proton abstraction from C-8, a nucleophile is generated that can attack C-1' of ribose with cleavage of the C-O bond activated by protonation by AB3. This sequence of connected events is stereoelectronically reasonable if the conformation of GTP adopted in this reaction is rotated about the N-9 - C-1' bond and leads to the formation of a reactive three-membered ring intermediate (figure 4). Stereoelectronic control of the course of enzyme catalysed reactions is well known especially in the context of catalysis mediated by pyridoxal phosphate.[18] Alternative mechanisms leading to three-membered ring can be written, for example involving an anion formed by deprotonation of N-3. In all cases, the reactive three-membered ring intermediate provides the route for the required side chain migration and ring expansion. In the case illustrated, ketonisation of the C-2' hydroxyl group with migration of hydride from C-2' to the adjacent apex of the three-membered ring affords the methylene group at C-7 of the pteridine. Concomitant

migration of the alkyl group to C-8 and opening of the three membered ring by breaking the N-9 C-8 bond affords the pyrazine ring of the pteridine.[21] All atoms are in the correct oxidation state for precursor Z which is formed by tautomerisation of the pteridine and its C-6 side chain and cyclisation of the side chain hydroxyl group to form a phosphodiester.

Although the incorporation observed by Rajagopalan was low, the degradation identifying the site of labelling was unambiguous.[7] We have therefore devised an alternative mechanism (figure 5) that correlates well with those proposed for the cyclohydrolases and their requirement for bases. In this alternative mechanism, a tautomer of the formamide intermediate adds nucleophilically to the carbonyl group of the formamide possibly via an enolate or enzyme-bound enamine. This reaction transfers C-8 of the purine to the side chain and permits its insertion into the side chain through a 1,2-acyl shift. In this way, the side chain is established in an oxidation state suitable for cyclisation to a dihydropterin and with the atoms in positions consistent with Rajagopalan's labelling experiments. Precedent is scarce for the 1,2-acyl shift but a superficially similar reaction that would lead to the correct labelling pattern has been described for base-catalysed rearrangements of α-hydroxyketones in ring D of androstanes;[22] This reaction is related to the well-known benzylic acid rearrangement. In the case of molybdopterin synthase, acid/base catalysis in particular protonating the acceptor carbonyl groups (figure 5) would be important.

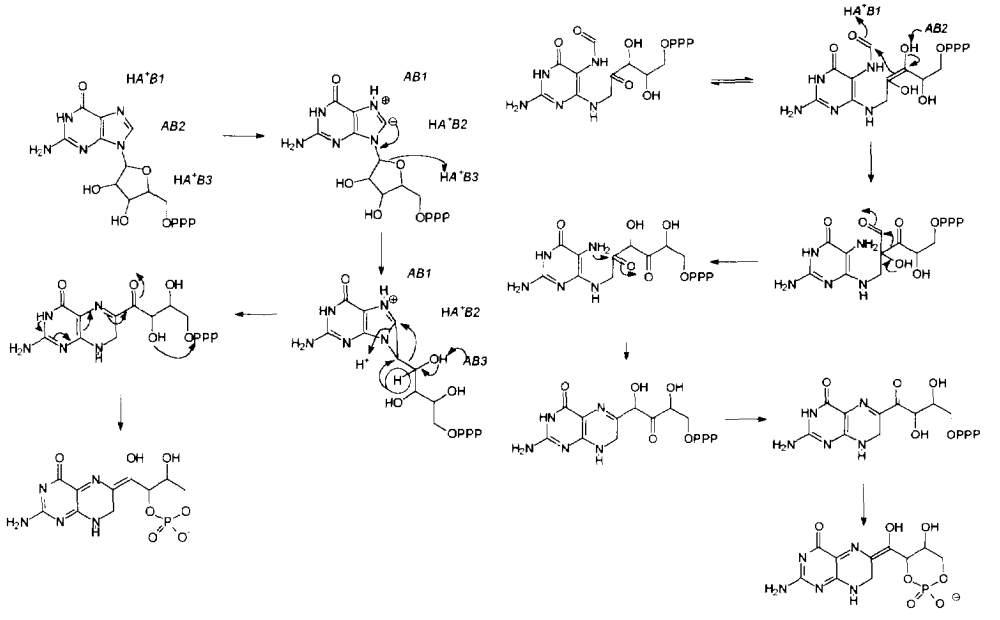


Figure 4 (left) and **figure 5** (right). Possible mechanisms for precursor Z formation from a GTP hydrolysis product

Concluding remarks. Taken together, these analyses of possible mechanisms for GTP-transforming mechanisms suggest that catalysis is plausible but unnecessary. All of the transformations can be satisfactorily interpreted through the action of two or three acid-base catalysts. The proposals suggest many experimental tests using kinetic methods, stereochemical probes, or enzyme inhibition studies[23] to improve our understanding of these important and fascinating enzymes.

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