ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 5825–5827

www.rsc.org/chemcomm

COMMUNICATION

The crystallographic structure of thermoNicotianamine synthase with a synthetic reaction intermediate highlights the sequential processing mechanism†

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Received 28th January 2011, Accepted 16th March 2011
DOI: 10.1039/c1cc10565e

We determined the three-dimensional structure of a complex between an archaeal nicotianamine synthase homologue and a chemically synthesised reaction intermediate. This structure suggests that the enzymes cavity allows both an ordered substrate binding and provides energetic coupling of the reaction intermediate formation and translocation.

Nicotianamine (NA; **1**) is a ubiquitous plant metal chelator involved in metal homeostasis.[1] It can bind different metals *in vitro*[2] thereby contributing to their distribution in the various organs of the plant. In addition, NA is the first intermediate in the biosynthesis of phytosiderophores and thus participates in iron acquisition by plants.[3] A decrease in the NA level causes various disorders in metal transport, which lead to defects in plant development and reproduction.[4] Conversely, an increase of the NA level in rice enhances the plant tolerance to iron and zinc deficiency but also renders it more sensitive to zinc, copper and nickel toxicity.[5] Recently, it was shown that NA enhances iron bioavailability to humans.[6]

NA is enzymatically synthesized by Nicotianamine synthase (NAS). This enzyme condenses three aminopropyl moieties of S-adenosylmethionine (SAM) and the autocyclization of one moiety leads to the formation of an azetidine ring. The precise catalytic mechanism of NA synthesis is still unknown but its main lines may be inferred from previous work on an archaeal homologue (MtNAS from *Methanothermobacter thermautotrophicus*). The X-ray structure of this enzyme was resolved as a complex with its product, which was identified as an analogue of NA and called thermoNicotianamine (tNA; **2**; Fig. 1).[7,8] The only difference between NA and tNA is the carboxy azetidine moiety of NA that is replaced by a glutamate moiety in tNA. Therefore, in addition to SAM, glutamate is a substrate for the archaeal homologue of NAS. The design and subsequent structure determination of a mutant (MtNAS-E81Q) resulted in an inactive apo-enzyme. The analysis of the three dimensional structures of this mutant complexed with both substrates (SAM and/or glutamate) suggested that the polymerization mechanism occurs at a single active site with stepwise translocation of the reaction intermediate (Fig. 1). The internal cavity of the enzyme is indeed composed of three subsites called S1, S2 and S3 with only the S1 subsite exposed to the solvent through a small inlet. According to the different X-ray structures, glutamate binds first in S1 but is translocated to S2 upon binding of SAM to the S1 site. After a first nucleophilic attack, a reaction intermediate (one aminopropyl moiety fused to glutamate; AP_2–Glu_1; **3**) is formed at the

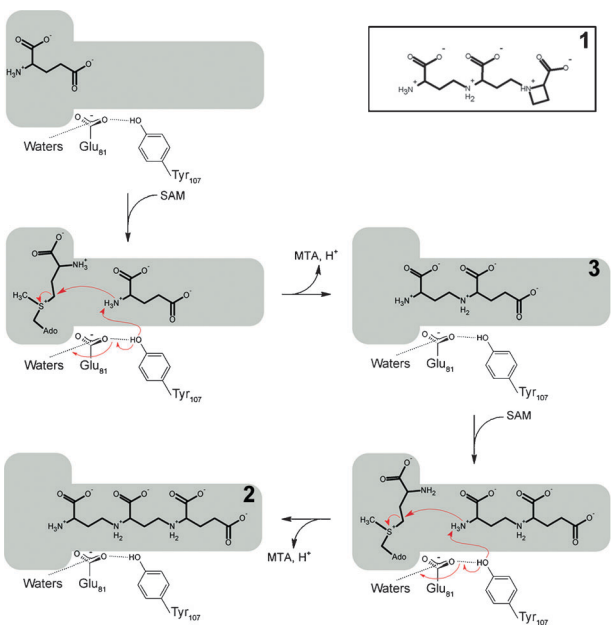

Fig. 1 Proposed reaction mechanism catalysed by MtNAS and the chemical structure of NA (**1**), tNA (**2**) and the reaction intermediate **3**. The cavity at the heart of the enzyme is depicted in grey.

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† Electronic supplementary information (ESI) available: Experimental details; a movie of the reaction catalysed by MtNAS. See DOI: 10.1039/c1cc10565e

S1–S2 subsites. Once synthesised, translocation of AP_2–Glu_1 to the S2–S3 subsites is required for the binding of a second molecule of SAM at the S1 subsite allowing a second nucleophilic attack that ultimately leads to the final tNA product. It is not clear whether this second translocation step occurred spontaneously or required the arrival of the novel SAM molecule, as is the case for glutamate.

In this communication, we report the synthesis of the reaction intermediate AP_2–Glu_1 (**3**) in three steps (Scheme 1), starting from commercially available *tert*-butyl glutamate. We then describe the three-dimensional structure of the complex with the MtNAS-E81Q mutant solved by the co-crystallization and molecular replacement method (Table S1, ESI†). This structure sheds new light on the exquisite mechanism of enzymatic synthesis of tNA.

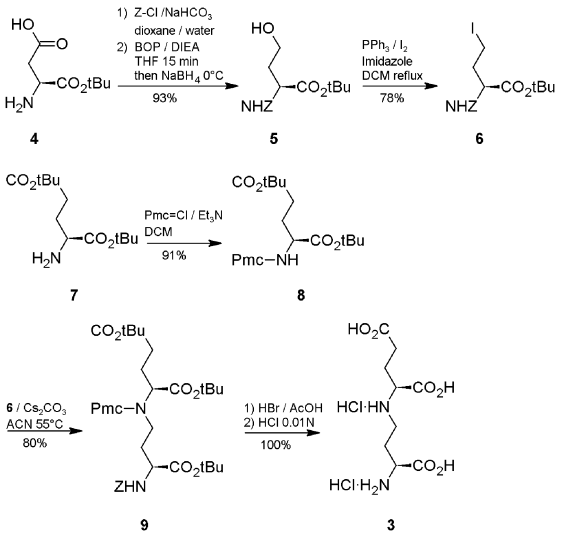
The synthetic approach was based on the assembly of two building blocks by *N*-alkylation:[9] an activated amine derived from glutamic acid in one part and an iodine synthon, a mimic of the aminopropyl moiety of SAM, on the other part.

tert-Butyl ester and Z protection were chosen for the iodine-containing compound benzyl 2-(*S*)-(*tert*-butoxycarbonylamino)-4-iodobutanoate (**6**). This key precursor for this strategy was prepared in three steps using L-*tert*-butyl aspartate (**4**) as a starting material (Scheme 1), like in a previously reported synthesis of azetidine.[10]

Compound **4** was treated with benzyl chloroformate under usual conditions ($NaHCO_3$, H_2O/dioxane) to afford the Z derivative, which was readily reduced using the McGeary conditions[11] (preactivation of the acid with BOP then reduction with $NaBH_4$) to afford the desired alcohol **5** in 93% yield. Then, the alcohol **5** was converted into the iodide **6** using iodine, triphenylphosphine and imidazole.[12]

Direct alkylation of the glutamic acid derivative **7** with compound **6** did not yield the desired product but only the starting material, proving the poor reactivity of the amine in this reaction and the necessity of a specific activation, which was achieved with a sulfonamide temporary protection.

Introduction of the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc)[13] protecting group on **7** led to the sulfonamide **8** with



Scheme 1 Synthesis of **6** and **3**.

90% yield (Scheme 1). This activation has been already used in the synthesis of reduced peptide bonds by the Mitsunobu reaction.[14] Sulfonamide **8** was then engaged in a reaction of *N*-alkylation with the compound **6** using caesium carbonate to give the protected precursor **9** with 80% yield. Removal of the Pmc, Z and *tert*-butyl protecting groups proceeded in a single step by treatment with 45% HBr in glacial acetic acid to afford quantitatively the reaction intermediate **3**.

Co-crystallization of the MtNAS-E81Q mutant with either compound **3** in the presence of SAM or with both substrates (SAM and glutamate) led to a ternary complex with bound products (E81Q-MtNAS–tNA–MTA; data not shown). This result demonstrates that the E81Q mutant is not entirely inactive, and also that the synthesised reaction intermediate is competent for the production of tNA. Compound **3** was then co-crystallized with the MtNAS-E81Q mutant and the three-dimensional structure of the complex was solved by molecular replacement. Inspection of the electron density in the cavity at the interface between the two domains of the protein clearly indicated the presence of **3** (Fig. 2). A total of ten hydrogen bonds contribute to its binding at the back of the cavity, in a conformation that is similar to what was found in the enzyme–product complex. Thus, interestingly, the reaction intermediate is not localized in S1–S2 subsites, the position where it is formed after the first nucleophilic attack, but at a translocated "final" position (S2–S3). In such a position, a second molecule of SAM can bind to the free S1 subsite for a second catalytic reaction to assemble the final product.

This study clearly confirms the proposed reaction mechanism, but also opens interesting questions related to the driving force controlling the translocation process. Indeed, in the structure of the MtNAS-E81Q–glutamate complex, the glutamate is stabilized in the S1 subsite and we showed that it is translocated deeper into the cavity (in S2 subsite) only upon SAM binding.[7] However, the present study shows that **3** is not located in the S1–S2 subsites but deeper at the back of the cavity, even in the absence of SAM. What is the rationale for the different behavior of the glutamate moiety and intermediate **3**? Part of the answer is that a larger number of hydrogen bonds stabilize the reaction intermediate in its final position,

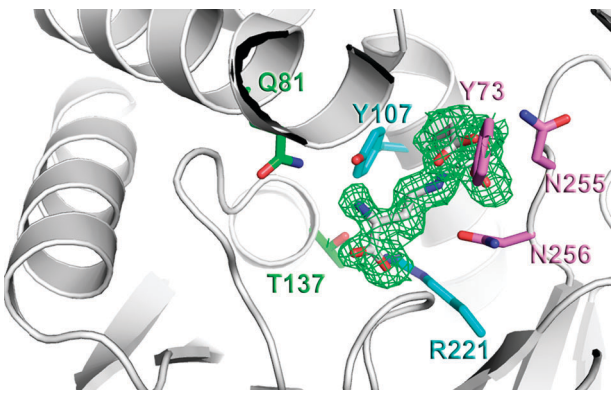


Fig. 2 Electron density omit map (F_o–F_c contoured at 2σ) of the reaction intermediate co-crystallized with MtNAS. Residues from subsites S1, S2 and S3 are depicted in green, blue and magenta, respectively. The reaction intermediate **3** is found in the S2 and S3 subsites.

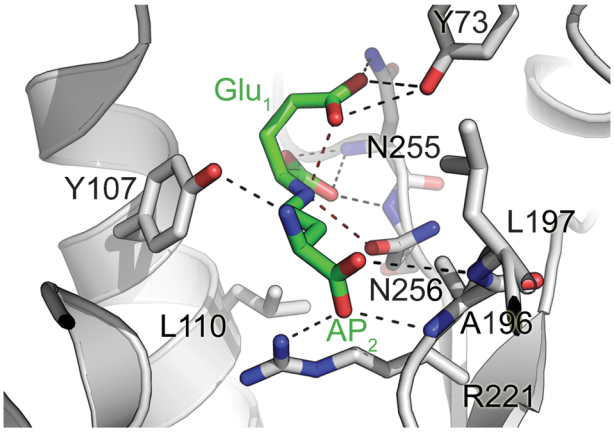


Fig. 3 Position of L110 and the secondary amine filter. Hydrogen bonds between the reaction intermediate (AP_2–Glu_1) and the residues in the cavity of MtNAS are depicted as dashed lines. Secondary amines are selected at the position of Glu_1 because primary amines would have a hydrogen atom pointing toward L110 that could not be stabilized.

compared with the bonds involved in stabilizing the glutamate in its initial position. However, this does not really explain why glutamate does not bind straightaway in S3. We believe that the reason could be the very different affinities of the S3 subsite for secondary and primary amines. Indeed, details of the interaction between the protein and the reaction intermediate show that the nitrogen atom of Glu_1 is involved in two hydrogen bonds (one with Oε1 of Asn256 and one intramolecular; Fig. 3). If one glutamate molecule were free to translocate to subsite S3, the position of the third hydrogen of its amine group would point toward the strictly conserved Leu 110. Because the side chain of this residue cannot engage any hydrogen-bond, primary amines are clearly destabilized at this position. Therefore, Leu110 plays a key role in the catalysis: by rejecting primary amines from the bottom of the cavity and admitting the secondary amine formed after the first catalytic step, it ensures an ordered substrate binding and controls the translocation process.

We now have snapshots of almost all the steps that lead to the formation of tNA by MtNAS. A realistic molecular movie can be generated that is based on experimental three-dimensional structures (ESI†, Movie 1). Although not comparable to much more complex enzymes such as the ribosome[15] or the non-ribosomal-peptide-synthase,[16] the case of MtNAS is interesting with regard to its processivity. Indeed, processive enzymes are defined as proteins that remain attached to their substrate and perform multiple catalytic cycles before dissociating.[17] In terms of the three-dimensional structure, processive enzymes display either grooves at their surface or symmetrical toroids that allow partial or complete substrate enclosure.[18] MtNAS is original as it uses a rigid frame harboring an internal asymmetrical cavity that precisely controls substrate and reaction intermediate processing and translocation.

This work is supported by the ANR (project HEMOLI; ANR-07-BLAN-0115-01) and the Commissariat à l'Energie Atomique (CEA). We thank J. Varlin and J. Lavergne for discussion. The coordinates for the MtNAS-E81Q–Reaction intermediate complex have been deposited in the Protein Data Bank (accession code 3O31).

Notes and references

1 C. Curie, G. Cassin, D. Couch, F. Divol, K. Higuchi, M. Le Jean, J. Misson, A. Schikora, P. Czernic and S. Mari, *Ann. Bot.*, 2009, **103**, 1–11.
2 (*a*) I. Benes, H. Ripperger and A. Kircheiss, *Experientia*, 1983, **39**, 261–262; (*b*) R. Rellan-Alvarez, J. Abadia and A. Alvarez-Fernandez, *Rapid Commun. Mass Spectrom.*, 2008, **22**, 1553–1562; (*c*) S. M. Reichman and D. R. Parker, *Plant Physiol.*, 2002, **129**, 1435–1438; (*d*) N. von Wiren, S. Klair, S. Bansal, J. F. Briat, H. Khodr, T. Shioiri, R. A. Leigh and R. C. Hider, *Plant Physiol.*, 1999, **119**, 1107–1114.
3 G. Weber, N. von Wiren and H. Hayen, *BioMetals*, 2008, **21**, 503–513.
4 M. Takahashi, Y. Terada, I. Nakai, H. Nakanishi, E. Yoshimura, S. Mori and N. K. Nishizawa, *Plant Cell*, 2003, **15**, 1263–1280.
5 S. Lee, U. S. Jeon, S. J. Lee, Y. K. Kim, D. P. Persson, S. Husted, J. K. Schjorring, Y. Kakei, H. Masuda, N. K. Nishizawa and G. An, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 22014–22019.
6 L. Zheng, Z. Cheng, C. Ai, X. Jiang, X. Bei, Y. Zheng, R. P. Glahn, R. M. Welch, D. D. Miller, X. G. Lei and H. Shou, *PLoS One*, 2010, **5**, e10190.
7 C. Dreyfus, D. Lemaire, S. Mari, D. Pignol and P. Arnoux, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 16180–16184.
8 C. Dreyfus, D. Pignol and P. Arnoux, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2008, **64**, 933–935.
9 M. Bouazaoui, J. Martinez and F. Cavelier, *Eur. J. Org. Chem.*, 2009, 2729–2732.
10 M. Bouazaoui, M. Larrouy, J. Martinez and F. Cavelier, *Eur. J. Org. Chem.*, 2010, 6609–6617.
11 R. P. McGeary, *Tetrahedron Lett.*, 1998, **39**, 3319–3322.
12 R. F. W. Jackson, R. J. Moore, C. S. Dexter, J. Elliot and C. E. Mowbray, *J. Org. Chem.*, 1998, **63**, 7875–7884.
13 (*a*) J. Green, O. M. Ogunjobi, R. Ramage, A. S. J. Stewart, S. Mccurdy and R. Noble, *Tetrahedron Lett.*, 1988, **29**, 4341–4344; (*b*) R. Ramage and J. Green, *Tetrahedron Lett.*, 1987, **28**, 2287–2290.
14 (*a*) K. Wisniewski and A. S. Kolodziejczyk, *Tetrahedron Lett.*, 1997, **38**, 483–486; (*b*) A. S. Kolodziejczyk, E. Sugajska, B. Falkiewicz and K. Wisniewski, *Synlett*, 1999, 1606–1608.
15 J. A. Dunkle and J. H. Cate, *Annu. Rev. Biophys. Biomol. Struct.*, 2010, **39**, 227–244.
16 A. Koglin and C. T. Walsh, *Nat. Prod. Rep.*, 2009, **26**, 987–1000.
17 P. H. Von Hippel, F. R. Fairfield and M. K. Dolejsi, *Ann. N. Y. Acad. Sci.*, 1994, **726**, 118–131.
18 W. A. Breyer and B. W. Matthews, *Protein Sci.*, 2001, **10**, 1699–1711.