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Product inhibition in the radical *S*-adenosylmethionine family

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ABSTRACT

Members of the radical *S*-adenosylmethionine (AdoMet) superfamily reductively cleave AdoMet to generate the highly reactive 5′-deoxyadenosyl radical (DOA·) which initiates biological transformations by abstraction of a hydrogen atom. We demonstrate that three members of the family: biotin synthase (BioB), lipoyl synthase (LipA) and tyrosine lyase (ThiH) are inhibited in vitro by a combination of the products 5′-deoxyadenosine (DOA) and methionine. These results suggest the observed inhibition is a common feature of the radical AdoMet proteins that form DOA and methionine as products. Addition of 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) to BioB, LipA or ThiH activity assays removed the product inhibition by catalysing the hydrolysis of DOA and gave an increase in activity.

1. Introduction

The radical *S*-adenosylmethionine (AdoMet) family of proteins contain a 4Fe–4S cluster and are dependent on AdoMet for activity [1]. The superfamily contains a conserved CxxxCxxC sequence motif [2] which co-ordinates three of the four iron atoms of the 4Fe–4S cluster [3,4]. The fourth iron has been shown to bind AdoMet, holding the sulfonium group in close proximity to the 4Fe-4S cluster [5] allowing the reductive cleavage of $[4Fe–4S]^{+}$-AdoMet to $[4Fe–4S]^{2+}$-Met and the 5′-deoxyadenosyl radical (DOA·). This highly reactive intermediate can abstract a hydrogen atom from a substrate to initiate subsequent radical chemistry [6–8] including functionalisation of sites that are usually regarded as unreactive. Some radical AdoMet proteins, such as lysine 2,3-aminomutase (KAM) [9] and spore photoproduct lyase (SPL) [10], utilise AdoMet as a co-factor which requires reversible cleavage of AdoMet and regeneration of DOA· in the reaction cycle. Many of the radical AdoMet proteins do not utilise AdoMet catalytically, instead using it as a substrate forming DOA and methionine as products.

Biotin synthase (BioB) and lipoyl synthase (LipA) are radical AdoMet proteins which insert sulfur atoms during the final step of the biosynthesis of biotin [11,12] and lipoyl groups [13,14]. In vitro activity assays have shown that biotin or lipoyl groups are produced in a stoichiometric ratio with the BioB or LipA proteins [15,16], probably due to depletion of the sulfur donor. In contrast in vivo studies have shown BioB can function catalytically (~20–60 biotin molecules formed per BioB) and the observed catalytic activity required 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN). It was found that MTAN could hydrolyse the glycosidic bond of DOA to yield adenine and 5′-deoxyribose [17,18], thus extending the substrate specificity of this enzyme which had previously been reported to utilise *S*-adenosylhomocysteine (SAH) and 5′-methylthioadenosine (MTA) [19]. The potential for product inhibition by DOA and methionine in vitro has only been investigated for a single example protein from the radical AdoMet family, biotin synthase (BioB). These experiments have yielded conflicting results: data from Ollagnier-de-Choudens et al. [20] indicated almost complete inhibition of BioB at a molar ratio of 1.5 DOA per BioB, corresponding to a DOA concentration of ~55 μM. However, this was not observed by Tse Sum Bui et al. [21] who reported that BioB was not inhibited by DOA.

Tyrosine lyase (ThiH) is member of the radical AdoMet family essential for thiamine biosynthesis in *Escherichia coli* and uses AdoMet as a substrate in order to cleave tyrosine (Fig. 1) [22,23]. Herein we report in vitro studies on the inhibitory effect of their common products, deoxyadenosine and methionine on three

Abbreviations: AdoMet, *S*-adenosylmethionine; BioB, biotin synthase; DOA, 5′-deoxyadenosine; DOA·, 5′-deoxyadenosyl radical; FldA, flavodoxin; Fpr, flavodoxin reductase; LipA, lipoyl synthase; MTA, 5′-methylthioadenosine; MTAN, 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase; SAH, *S*-adenosylhomocysteine; ThiH, tyrosine lyase

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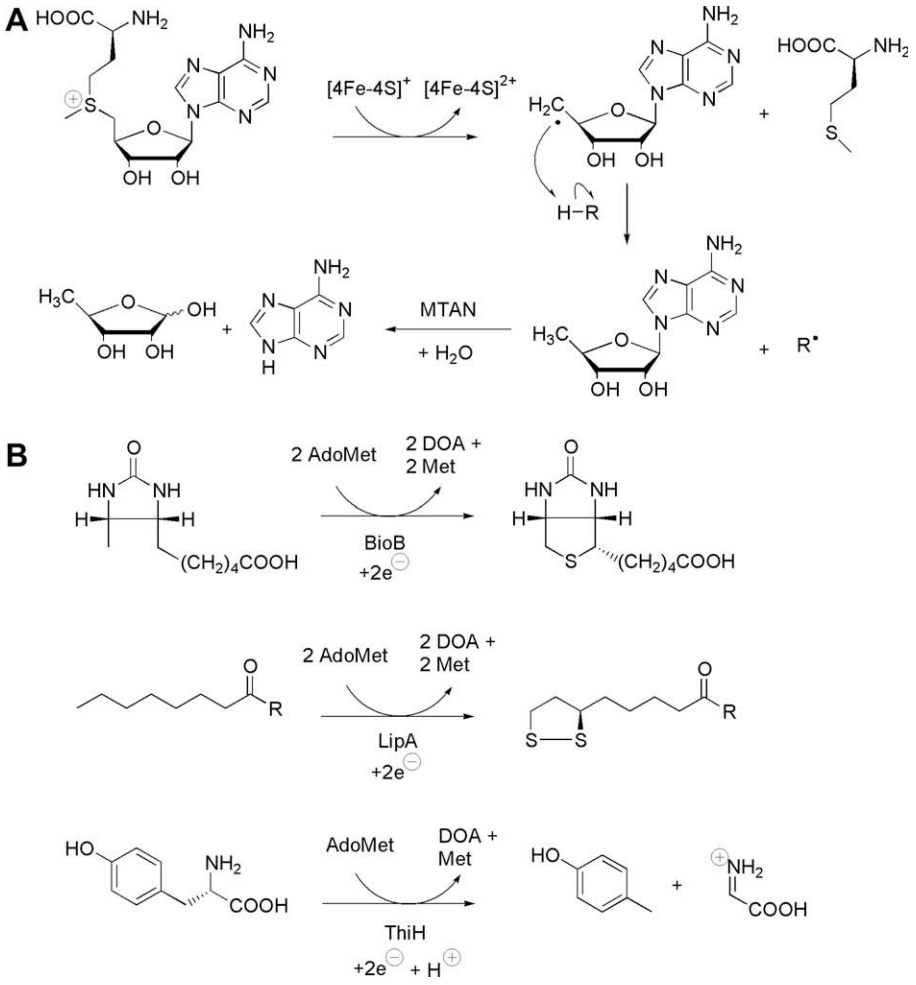

Fig. 1. (A) Reductive cleavage of AdoMet and subsequent metabolism of DOA by MTAN. (B) The biotransformations requiring BioB, LipA and ThiH.

radical AdoMet proteins: BioB, LipA and ThiH. These products show cooperative inhibition which can be overcome by the presence of MTAN. This mode of inhibition is likely to be general for the radical AdoMet proteins which utilise AdoMet as a substrate and can be circumvented by the removal of DOA by MTAN mediated hydrolysis.

2. Materials and methods

2.1. Protein expression and purification

The plasmids pet6H:bioB and pProExHTApfs were generous gifts from Dr. D. Campopiano (University of Edinburgh, UK) and Prof. K. Cornell (Oregon Health Science University, Portland), respectively. BioB [24], LipA [25], ThiGH, flavodoxin (FldA), flavodoxin reductase (Fpr) [23] and MTAN [26] were expressed and purified as described previously and where necessary, the iron sulfur clusters chemically reconstituted and the iron content analysed as described by Fish [27].

2.2. In vitro assays with radical AdoMet proteins

All manipulations were carried out in an anaerobic glovebox (Belle Technology) maintained at <1 ppm O_2. Activity assays and the HPLC analysis of reaction products were modifications of methods described previously [16,23,25] and are described in detail in Supplementary data. The activity was measured by monitoring the production of biotin for BioB, lipoyl plus dihydrolipoyl tripeptide for LipA and *p*-cresol for ThiH. To investigate product inhibition, the reaction products (DOA, methionine or DOA plus methionine) were added to activity assays in the following concentration ranges: ThiH, all 0–1 mM; LipA, all 0–1 mM; BioB, methionine 0–700 μM, DOA 0–800 μM, DOA plus methionine 0–400 μM. To investigate the alleviation of product inhibition, in vitro assays were further supplemented with MTAN (10 μM).

2.3. MTAN activity assay

The MTAN activity assay contained MTAN (5 nM), DOA (12.5–800 μM), ammonium acetate (20 mM, pH 7.5) and BSA (0.1 mg mL^{-1}). To estimate the K_M for DOA, assays were initiated in parallel in a 96 well PCR plate incubated at 37 °C. Assays were initiated by the addition of MTAN and stopped at time points (0–5 min) by the addition of perchloric acid (1% v/v). The precipitated protein was removed by centrifugation, supernatants (100 μL) neutralized with ammonium hydroxide and analysed by RP-HPLC using a Gemini C_{18} (4.6 × 250 mm, 5 μm, 100 Å) reverse phase HPLC column (Phenomenex). The mobile phase (0.7 mL/min) was an initial 5 min isocratic phase of 5% acetonitrile in 20 mM ammonium acetate, pH 6.0, followed by a 10 min linear gradient to 50% acetonitrile. Standards of adenine and DOA had retention times of 10 min and 13 min, respectively, and were used to construct a calibration curve to quantify the concentration of DOA and adenine in activity assays.

3. Results

3.1. Inhibition by DOA and methionine

To investigate the possibility of the AdoMet cleavage products in inhibiting radical AdoMet protein activity, increasing amounts of each product was added to activity assays. Relative activity was calculated by comparison with a standard assay to which no products had been added. Methionine had a very weak inhibitory effect on BioB and LipA, but no effect was observed on ThiH, even at a concentration of 1 mM, which is far greater than might accumulate in a typical in vitro assay (Fig. 2A). DOA was found to be a weak inhibitor of all three proteins (Fig. 2B) and 1 mM of DOA only resulted in 50–60% inhibition. As DOA and methionine are produced in equimolar amounts, their potential to inhibit in a cooperative manner was investigated. The addition of a combination of DOA and methionine to assays resulted in a greater degree of inhibition than the individual compounds in all three cases (Fig. 2C). The biotin forming reaction was particularly sensitive, being completely inhibited at 400 μM of DOA and methionine. The inhibition could be fitted to a three parameter logistic sigmoid to give the apparent IC_{50} for BioB, LipA and ThiH under the assay conditions of 114 ± 14, 249 ± 22 and 445.6 ± 35 μM, respectively (Fig. 2C).

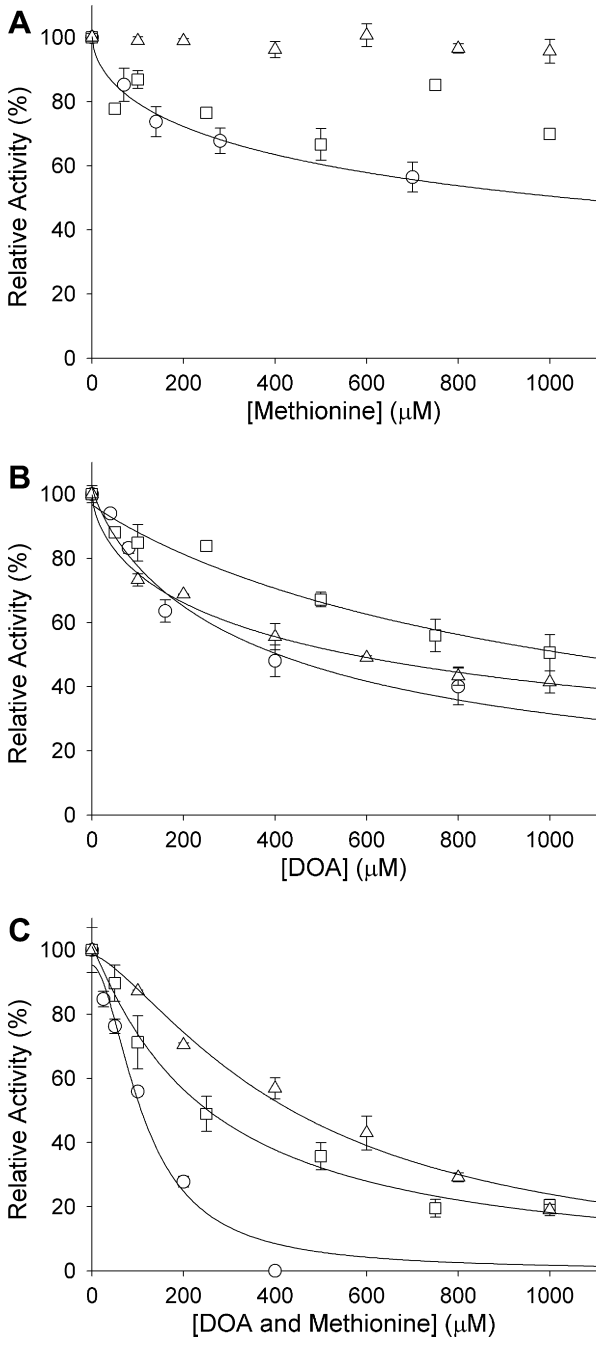

Fig. 2. The effect of (A) methionine, (B) DOA and (C) equimolar amounts of DOA and methionine on BioB (open circles), LipA (open squares) and ThiH (open triangles). The data for the effect of methionine on LipA and ThiH could not be fitted.

3.2. Kinetic analysis of DOA as a substrate for MTAN

The products of the MTAN-catalysed hydrolysis of DOA were confirmed using ^{1}H NMR analysis (Supporting data) [18]. To further understand the substrate diversity of MTAN, we investigated the kinetic parameters of this enzyme with DOA as the substrate using the rates of reaction at seven substrate concentrations (Fig. 4). MTAN activity assays were analysed using HPLC to monitor the disappearance of DOA and the formation of adenine (Fig. 3). The kinetic parameters of MTAN are reported in Table 1.

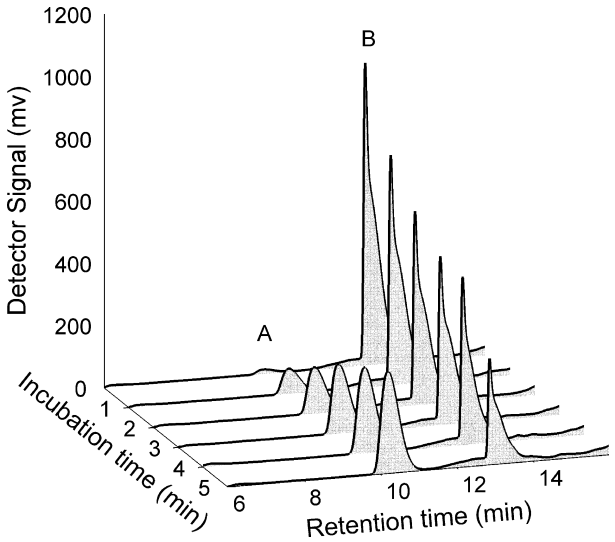

Fig. 3. HPLC analysis of MTAN activity. Adenine (peak A) and DOA (peak B) were detected by monitoring the absorbance at 254 nm.

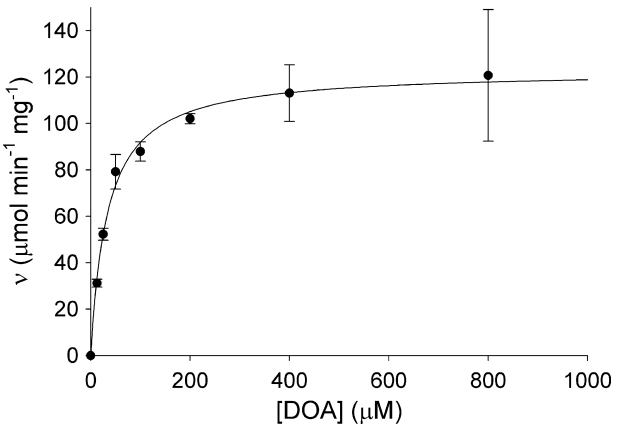

Fig. 4. Michelis–Menten plot for MTAN with 5′-DOA as the substrate.

Table 1
Kinetic constants of MTAN. Data for MTA and SAH were derived from Della Ragione et al. [32].

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_M (μM)	k_{cat} (s^{-1})	Catalytic efficiency (M^{-1} s^{-1})
DOA	120 ± 3	33 ± 3	49	1.5×10^6
MTA	370	0.40	150	370×10^6
SAH	No data	4.3	No data	No data

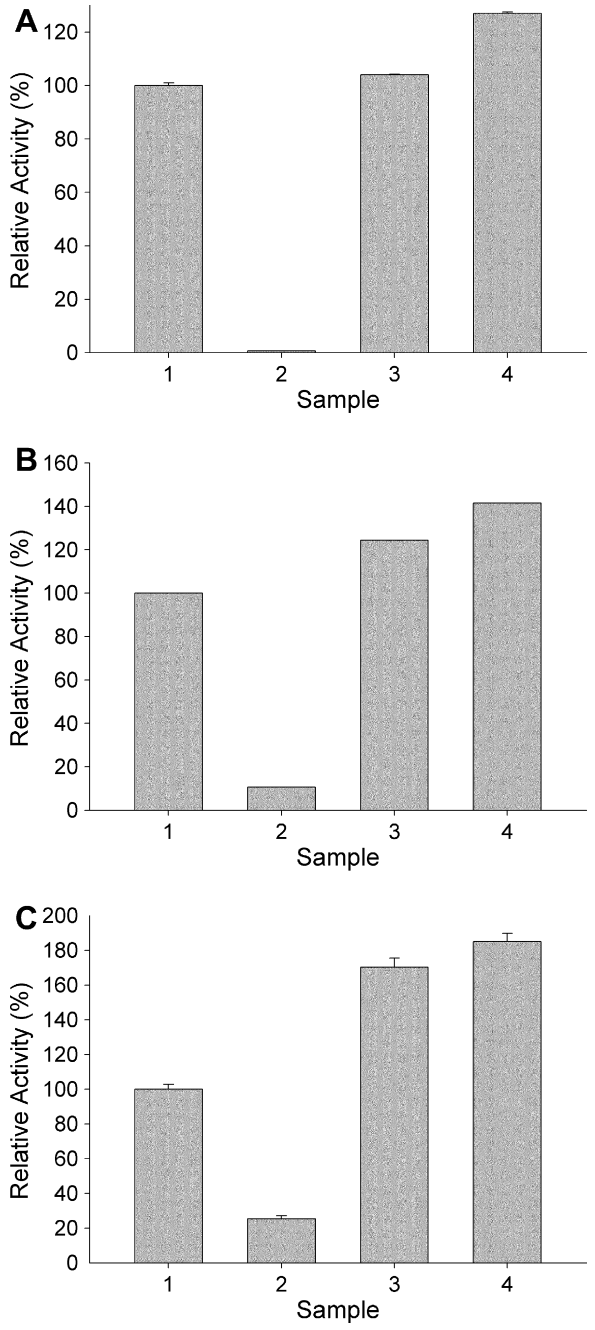

Fig. 5. Effect of adding MTAN to radical AdoMet activity assays. (A) BioB; (B) LipA and (C) ThiH. Assays were supplemented with the following additional reagents: sample 1, no further additions; sample 2, DOA and methionine (400 μM of each for BioB; 1 mM of each for LipA and ThiGH); sample 3, MTAN (10 μM), DOA and methionine (400 μM of each for BioB; 1 mM of each for LipA and ThiGH); and sample 4, MTAN (10 μM).

3.3. Removal of reaction products

Having demonstrated that MTAN can rapidly hydrolyse DOA we sought to test it as a potential solution to the observed cooperative inhibition of all three radical AdoMet proteins by methionine and DOA. Therefore, we investigated the effect of adding MTAN to in vitro activity assays that had been doped with inhibitory concentrations of DOA and methionine. After incubation at 37 °C, the comparison of activity assays with and without MTAN shows that MTAN restored the activity of all three radical AdoMet proteins (Fig. 5, sample 2). The activity in the MTAN containing samples slightly exceeds the observed activity of the positive control (Fig. 5, samples 1 and 3), due to the hydrolysis of the DOA formed during the reaction. This conclusion is supported by the observation that the addition of MTAN to an activity assay from the beginning of the reaction resulted in a reproducible enhancement of activity for BioB, LipA and ThiH (Fig. 5, sample 4).

4. Discussion

Radical AdoMet proteins can be divided into two groups, based on whether they use the deoxyadenosyl radical catalytically or stoichiometrically [28]. The enzymes that utilise the DOA radical catalytically are unlikely to accumulate enough DOA or methionine to reach inhibitory concentrations. However, the majority of family members characterized thus far have been shown to form DOA as a product that is released from the active site at the end of each catalytic cycle. The data presented in this study shows the most efficient inhibition of all three radical AdoMet enzymes occurred when both DOA and methionine were present in equimolar amounts. The apparent IC_{50} values determined from these experiments suggest that inhibition by DOA and methionine has a significant effect during in vitro assays. It seems likely that the pattern of inhibition observed for LipA, BioB and ThiH will be general across this sub-family and the accumulation of DOA and methionine during in vitro assays will lead to a decrease in observed activity. A particularly susceptible sub-class is formed by those radical AdoMet proteins (such as BioB and LipA) that use two equivalents of AdoMet to cleave two C-H bonds in order to complete their biosynthetic function as they must generate two equivalents of DOA and methionine.

BioB and LipA are thought to be limited to forming one equivalent of biotin or lipoyl moiety in vitro due to the depletion of the *holo*-protein derived sulfur source [16,21,29]. The addition of the nucleosidase MTAN does not alter this constraint, but MTAN addition does relieve the cooperative inhibition of all three proteins (BioB, LipA and ThiH) by DOA and methionine. These results are consistent with the suggestions of Choi-Rhee and Cronan [18] that variable amounts of MTAN in BioB preparations could account for variations in ability of DOA to inhibit BioB. Furthermore, the addition of MTAN results in a significant increase in the amount of product formed (Fig. 5), as any potentially inhibitory DOA formed during the reaction is hydrolysed in situ. This may represent a useful general approach applicable to the large number of radical AdoMet proteins that are currently subject of mechanistic investigation [1]. The absence of product inhibition will simplify kinetic analysis as well as increase the observed rates of reactions.

The intracellular concentration of methionine in *E. coli* has been estimated at 100 μM [30]. The observed cooperative inhibition of radical AdoMet proteins suggests that the in vivo formation of DOA would result in inhibition of the network of pathways containing radical AdoMet proteins. The presence of the nucleosidase MTAN that hydrolyses deoxyadenosine is postulated to ensure that DOA does not reach inhibitory concentration. Consistent with this hypothesis, deletion mutants lacking *pfs* (the gene encoding

MTAN) were observed to show retarded growth [18] that could be partially restored by the addition of biotin and lipoic acid. MTAN has been identified as a potential antibiotic target [31] because of its involvement in quorum sensing. However, it may be that inhibition of MTAN has the unanticipated benefit of significantly decreasing the activity of more that one radical AdoMet enzyme that is essential for bacterial growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.044.

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