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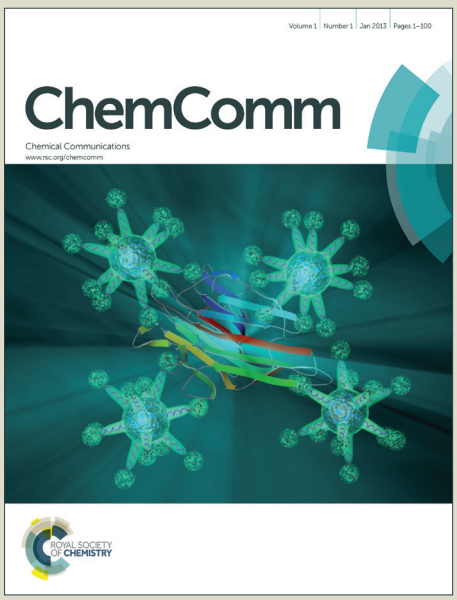

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Rationally engineered variants of *S*-adenosylmethionine (SAM) synthase: reduced product inhibition and synthesis of artificial cofactor homologues

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M. Dippe, W. Brandt, H. Rost, A. Porzel, J. Schmidt, and L. A. Wessjohann*[a]

_S_-adenosylmethionine (SAM) synthase was engineered for biocatalytic production of SAM and long-chain analogues by rational re-design. Substitution of two conserved isoleucine residues extended the substrate spectrum of the enzyme to artificial _S_-alkylhomocysteines. The variants proved to be beneficial in preparative synthesis of SAM (and analogues) due to a much reduced product inhibition.

S-adenosylmethionine synthase (SAMS, E.C. 2.5.1.6) is a key enzyme in biological methyl transfer and catalyzes the formation of *S*-adenosylmethionine (SAM) from *L*-methionine and ATP (Fig. 1). Since only very few enzymes rely on other donors with reactive methyl groups,[1-4] SAM serves as the general cofactor for cellular methyltransferases in all organisms.[1,2,5] Thus, SAM is involved in a multitude of biological processes such as DNA modification[6] or neurotransmitter metabolism in brain.[7] In plants, it is required for major metabolic pathways like lignin biosynthesis [8] and also for the modification of secondary metabolites.[1-3]

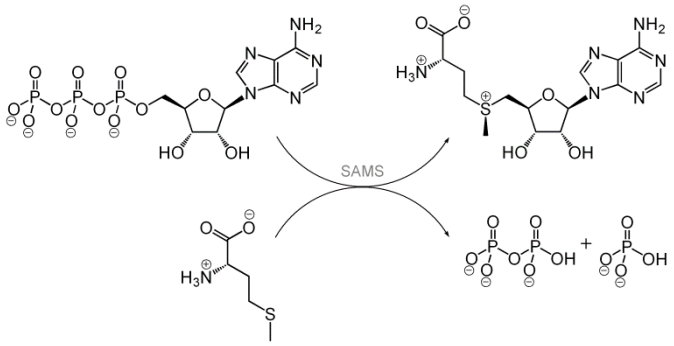

Fig. 1. Scheme of the SAMS reaction. From the two substrates, ATP and *L*-methionine (left), SAM (right, top) is produced. The co-products phosphate and diphosphate (right, below) originate from the triphosphate moiety of ATP.

In particular, phenylpropanoids, isoprenoids or alkaloids are often decorated by a specific methylation pattern which is essential for their biological activity.[1,3] Hence, synthetic routes to these valuable compounds often necessitate at least one methylation step. In many of these reactions, enzymatic methyl transfer has proven to be superior to traditional chemical methods because of strict chemo- and regioselectivity. However, the application of methyltransferases is still limited since the biologically active epimer of their costly cofactor SAM is hardly accessible, i.e. too expensive if produced by chemical synthesis. Thus, methylation by living whole-cell biotransformation is a promising alternative. The methyl acceptor is added to a suspension of microorganisms which express the recombinant enzymes and provide SAM from internal or fed *L*-methionine. However, this strategy has two major drawbacks. The intracellular concentration of endogenously available SAM often can be flux-determining for subsequent methyl transfer reactions, leading to slow or incomplete methylation. This is relevant in producer strains requiring SAM in high quantity. This limitation might be partially circumvented by over-expression of the SAMS enzyme of the expression host,[9] or by (multiple) integration of a recombinant enzyme with higher activity. However, commonly SAMS enzymes are not suitable for this strategy due to low specific activity or inhibition by their product SAM, thus limiting its own production in vivo and in vitro, despite sufficient availability of substrates and biocatalyst.[10,11]

In addition, this type of metabolic engineering does not allow transfer of alkyl chains other than methyl residues. Most eubacterial SAMS enzymes do not tolerate changes in the structure of their amino acid substrate, and thus they lack the ability to synthesize cofactor homologues or analogues from artificial methionine derivatives. From the large number of those SAMS enzymes which have been characterized, only two proteins tolerably convert *S*-ethyl-*L*-homocysteine (ethionine) to *S*-adenosylethionine.[11,12] In contrast, an increasing number of enzymes from archaea and mammals were recently found to convert a broad range of non-natural substrates, e.g. those from *Sulfolobus solfataricus*,[13] *Methanocaldococcus jannaschii*[14] or the human isoform MAT2A.[14] These enzymes proved to be useful tools in the synthesis of SAM derivatives from methionine homologues and the corresponding selenium compounds.[13,14] The molecular basis of this unusual promiscuity, however, is still subject of debate. Ten structures of SAMS (including enzymes from eubacterial, archaeal and mammalian origin) have been published so far,[13] implicating that relaxed substrate specificities might be related to the architecture and

dimension of the active site[13,15] and/or to interaction with a flexible neighboring loop region upon substrate binding.[13,16,17] Due to this indeterminancy in structure-activity relationships, studies on engineering of SAMS enzymes for activity, substrate specificity or product inhibition are scarce.[15,18]

Based on the recent informations from literature, x-ray and homology models (v.i.), we believed to have a basis for the rational re-design of a SAMS to produce a practically useful variant suitable for synthesis of SAM homologues. The SAMS from *Bacillus subtilis* (strain ATCC 6051) was chosen because this microbial protein has been shown to be efficiently produced in *Escherichia coli*.[19] Accordingly, high yields (59 mg l^{-1} of culture) of active and chromatographically homogeneous protein (see Fig. S1 in the Supporting Information) could be obtained after affinity purification. The enzyme was active towards the L-enantiomer of methionine, and towards a racemic mixture of both stereoisomeric forms of the amino acid. The reaction rates as a function of the substrate concentration (Fig. 2a) are characterized by sigmoid kinetics, which can be well described by the Hill function. The resulting kinetic parameters are shown in Table S1 in the Supplementary Information. The protein specifically converted the *L*-enantiomer of methionine because the concentration of this substrate at half-maximum saturation ($S_{0.5}$) was nearly twofold lower than that for *D*,*L*-methionine. However, the maximum rates (V_{max}) and Hill coefficients (*h*) observed for *L*-methionine and racemic methionine were similar. Therefore, *D*-methionine is not inhibitory to SAMS.

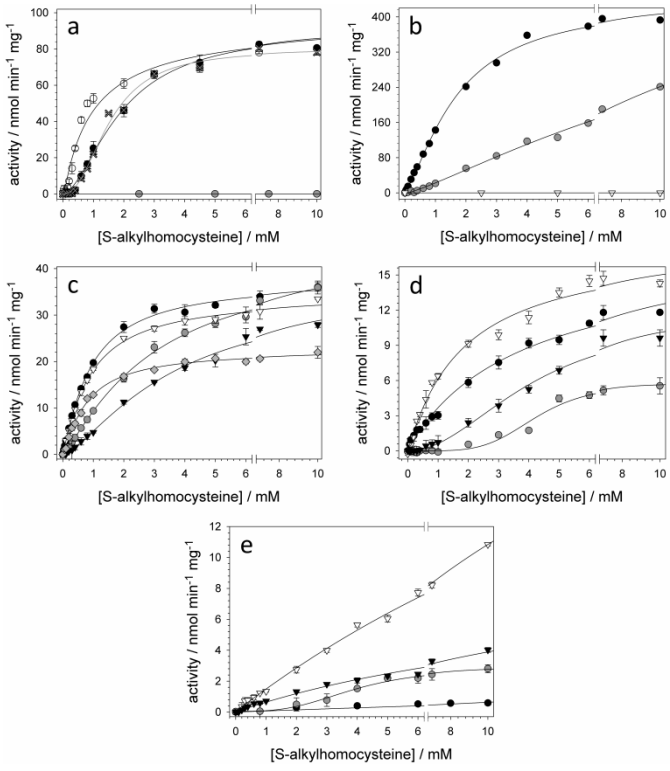

Fig. 2. Rates of conversion of methionine and derivatives catalyzed by the SAMS enzyme from *B. subtilis* (a), and its variants I317V (b), I317A (c), I105V/I317A (d) or I105A/I317A (e) as a function of the substrate concentration. The reaction with *L*-methionine (○), *D*,*L*-methionine (●), *D*,*L*-methionine-(methyl-D_3) (×), *D*,*L*-ethionine (●), *S*-n-propyl-*D*,*L*-homocysteine (▽), *S*-n-butyl-*D*,*L*-homocysteine (▼) or *S*-(prop-1-en-1-yl)-*D*,*L*-homocysteine (◆) was assessed by determination of phosphate released from the co-substrate ATP during the reaction. Curves were fitted according to the Hill model.

The enzyme was also tested for conversion of a series of methionine derivatives (Fig. 3) which differ from the natural substrate by introduction of deuterium atoms (**1**), in the length of their linear (**2** – **4**) or branched (**5** – **7**) thioether-bound alkyl chain as well as in functionalization by double bonds (**8**, **9**) or hydrophilic groups (**10**, **11**). As the enzyme was comparably active towards *D*,*L*-methionine-(methyl-D_3) (Fig. 3, **1**) and non-deuterated methionine (Fig. 2a), it proved to be well suitable for the production of isotope-labeled SAM. Similar to other eubacterial SAMS enzymes, none of the other artificial amino acids was accepted as substrate. This result could be explained by a model of the tertiary structure of the enzyme which is based on the crystal structure of the homologous protein from *E. coli*.[16] Fig. 4a shows the ternary complex of the protein with its product SAM. A detailed view of the bound SAM (Fig. 4b) indicates that it is recognized by the formation of several hydrogen bonds with the enzyme. On a closer inspection of this binding site the two isoleucines I105 and I317 appeared as residues which directly interact with the methyl group of SAM. Therefore, it seems likely that these residues disturb product formation from methionine derivatives with more bulky substituents. However, both isoleucines do not seem to be restrictive for the accommodation of the artificial amino acid substrate in the active site (Fig. 4c) prior to adenosylation, which indicates a conformational shift of the protein during catalysis. In conclusion, the isoleucine residues which are conserved in enzymes from eubacteria, but also in the majority of archaeal and eukaryotic proteins (see Fig. S2 in Supplementary Information) may largely contribute to the inability of many SAMS to convert extended methionine derivatives.

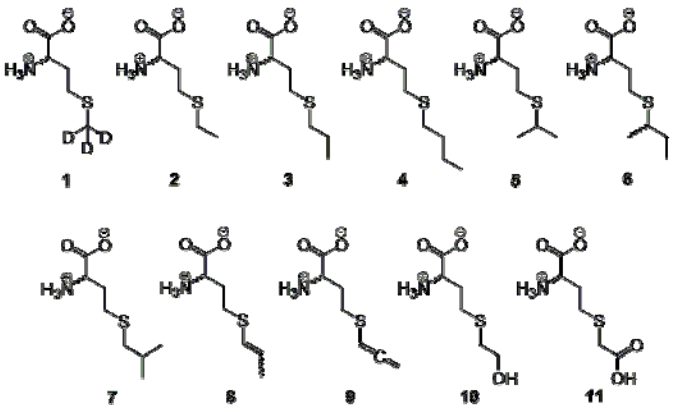

Fig. 3. *S*-Alkylhomocysteines used in this study. **1** *D*,*L*-methionine-(methyl-D3), **2** *D*,*L*-ethionine, **3** *D*,*L*-n-propionine, **4** *D*,*L*-n-buthionine, **5** *D*,*L*-isopropionine, **6** *S*-(1-methylpropyl)-*D*,*L*-homocysteine, **7** *S*-(2-methylpropyl)-*D*,*L*-homocysteine, **8** *S*-(prop-1-enyl)-*D*,*L*-homocysteine, **9** *S*-(propa-1,2-dienyl)-*D*,*L*-homocysteine, **10** *S*-(2-hydroxyethyl)-*D*,*L*-homocysteine, **11** *S*-(2-carboxymethyl)-*D*,*L*-homocysteine.

To prove this hypothesis, the two positions were exchanged against less voluminous residues by site-directed mutagenesis. First, the residue I317 which has the lowest distance to the methyl group of the SAM product (3.7 Å) was substituted to valine. The resulting enzyme variant shows a markedly enhanced activity towards *L*-methionine and *D*,*L*-methionine (Fig. 2b). The $S_{0.5}$ values for both substrates (Table S1 in Supporting Information), however, were similar to the wild-type protein. Due to its more spacious active site, the variant was also tolerably active (Fig. 2b) towards *D*,*L*-ethionine (**2**). In a second variant, I317 was exchanged by the much less voluminous alanine. Accordingly, the enzyme accepted an even broader spectrum of substrates (Fig. 2c) including methionine,

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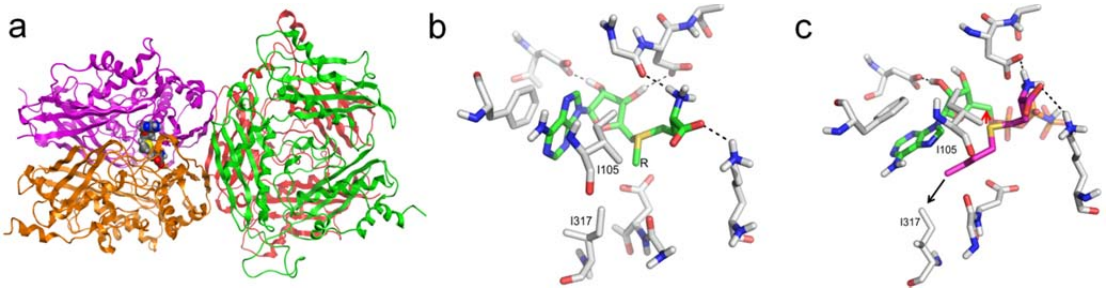

Fig. 4. Model of the SAM synthase from *B. subtilis.* a) Tertiary structure of the tetramer with SAM (space fill representations) bound between two monomer units. b) Active site with the bound product SAM (green carbon atoms). The space for docking of SAM derivatives with larger functional groups R is restricted by the two isoleucines I105 and I317 which were therefore subject of site-directed mutagenesis. c) Arrangement of the substrate analogues *S*-*n*-butyl-*L*-homocysteine (magenta carbon atoms) and adenylyl imidodiphosphate (green carbon atoms) in the active center. The arrows indicate the nucleophilic attack (red arrow) during catalysis. The reaction is accompanied with conformational change of the protein, which will lead to a steric clash of the butyl with the isoleucine-317 side chain (black arrow).

ethionine and the bulky homologues *S*-propyl- and *S*-butyl-homocysteine (**3** and **4**, resp.)†. The conversion of all substrates shows almost identical maximum rates (Table S1 in Supporting Information). Even the unsaturated substrate *S*-(prop-1-enyl)-*D*,*L*-homocysteine (**8**) was accepted by the enzyme variant (Fig. 2c).

Despite its relaxed substrate specificity, the enantioselectivity of the variant towards the amino acid substrate was similar to that of the wild-type enzyme, e.g. in the conversion of methionine (as judged from $S_{0.5}$ values for *L*- and racemic methionine, see Table S1 in Supporting Information). Likewise, from both stereoisomers of buthionine the substrate with *L*-configuration was exclusively converted (21.2 ± 0.1 nmol min^{-1} mg^{-1} at a substrate concentration of 5 mM).To further probe the role of the proximate isoleucine residue I105 in substrate recognition, the variants I105V/I317A and I105A/I317A were generated. With increasing size of the active center of the enzymes, conversion of the natural substrate methionine (Fig. 2d, e) is progressively reduced. On the other hand, the variants prefer long-chain substrates. Compared to the wild-type enzyme (Fig. 2a), substrate specificity of the variant I105A/I317A (Fig. 2e) is inverted (*S*-propylhomocysteine > *S*-butylhomocysteine > ethionine >> methionine). Hence, steric effects play a major role in substrate conversion by SAMS enzymes, and selectivity for certain substrates can be engineered by exchange of two amino acid positions only. However, unlike for the promiscuous SAMS enzyme from *Sulfolobus solfataricus*[13], activity of the variants towards methionine analogues with increased steric requirements -- such as branched-chain (**5** – **7**) or polyunsaturated (**9**) compounds -- was marginal (≥ 0.3 nmol min^{-1} mg^{-1}). Likewise, derivatives having a hydroxyl (**10**) or carboxyl (**11**) group were not accepted‡.

Subsequent to enzyme characterization, the suitability of the wild-type protein and of variants with appropriate substrate specificity (I317V and I317A) for the synthesis of SAM and analogues was evaluated by high-performance thin-layer chromatography (see Fig. S3 in the Supporting Information). Since the enzymes were not inhibited by the *D*-enantiomer of the amino acid substrates (Table 1), methionine and its derivatives could be advantageously applied as racemic mixture of its stereoisomeres in these reactions. The time course of SAM formation by the wild-type enzyme is shown in Fig. 5a. As described for several other SAMS proteins, the enzyme is inhibited by its product SAM[10,11,20] which results in stagnating conversion and low yield. In contrast, this product inhibition is completely reduced in the variant I317V (Fig. 5b) and less pronounced in case of the mutant enzyme I317A (Fig. 5c).

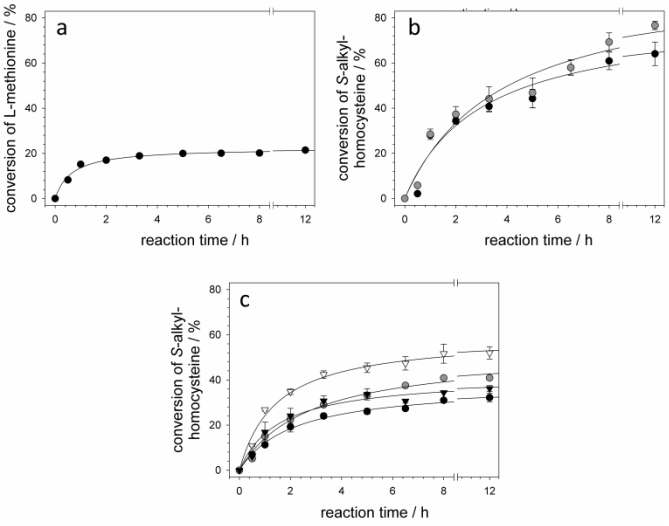

Fig. 5. Formation of SAM and analogues by SAMS enzyme from *B. subtilis* (a), or its variants I317V (b), and I317A (c). The conversion of racemic methionine (●), ethionine (◉), *S*-propyl-homocysteine (▽) and *S*-butylhomocysteine (▼) was performed in the presence of equal amounts of substrate (10 mM) and enzyme (10 mU ml^{-1}). The reactions were analyzed by HPTLC as described in the Supplementary Information.

Accordingly, transformation of methionine and ethionine by SAMS-I317V in preparative synthetic reactions led to high conversion rates for the amino acid substrates. The syntheses were performed similar to the kinetic experiments but in larger scale (200 µmol). After prolonged incubation (18 hours) to reach maximum product yield, 84 % and 89 % of the *L*-amino acid was converted. In the production of the n-propyl- and n-butyl analogues of SAM by the variant I317A, 43 % and 28 % of conversion was reached after 8 hours.
After product purification by cation exchange chromatography, SAM and its homologues could be isolated in final yields of 25 % (Me), 17 % (Et), 8 % (Pr) and 11 % (Bu) based on racemic starting material. As proven by ^{1}H NMR, the enzymatically produced SAM contained a high excess (≥ 90 %) of the biologically active (*S*,*S*)-epimer. Interestingly, preparations of the *S*-adenosyl-*L*-ethionine, -propionine and -buthionine were diastereomeric mixtures, racemic with respect to the chiral sulfonium center. As described above, the enzymatic reaction is stereoselective, and thus we assume that the faster epimerisation of higher homologues is a result of the higher +I effect and the purification process.

Conclusions

In conclusion, a detailed study of the active site of *S*-adenosylmethionine synthases and docking of substrates and products allowed the targeted introduction of subtle variation of size and hydrophilicity of the methionine binding site. This resulted in dramatic changes in activity and in an altered substrate scope. Most of all it gave an entry into enzymes with reduced or almost absent product inhibition. SAMS variants suitable for larger scale application in synthesis and biotechnology are now available.[20]

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Notes and references

[a] Leibniz-Institute of Plant Biochemistry, Department of Bioorganic Chemistry, Weinberg 3, D-06120 Halle, Germany
wessjohann@ipb-halle.de

† The influence of position 317 on catalytic turnover and its crucial role in determination of substrate spectrum was additionally confirmed by introduction of other small- and medium-sized residues. Enzymes substituted for aliphatic (I317G, I317P, I317L) or polar (I317E, I317D, I317N) amino acids show a strongly reduced activity (≥ 5.4 nmol min^{-1} mg^{-1}). On the other hand, substitution by cysteine resulted in an active enzyme which – similar to the variant I317A – converted methionine and homologues (59.4 ± 0.4, 16.4 ± 0.7, 8.4 ± 0.3 and 6.0 ± 0.3 nmol min^{-1} mg^{-1} for conversion of 5 mM D,L-methionine, -ethionine, -propionine and -buthionine, respectively).

‡ Engineering of specificity towards these hydrophilic compounds by introduction of one (variant I105V/I317S) or two (variant I105S/I317S) serine residues into the hydrophobic amino acid binding site was also not successful and led to completely inactive enzymes (data not shown).

Electronic Supplementary Information (ESI) available: see DOI: 10.1039/c000000x/

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