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Overcoming Product Inhibition of *S*-Adenosyl-L-methionine (SAM) Synthetase: Preparation of SAM on the 30 mM Scale.

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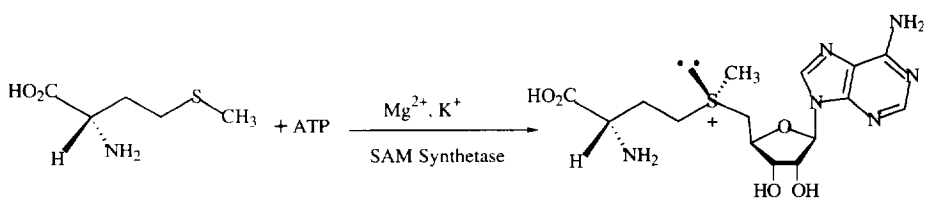
Abstract: Product inhibition of SAM synthetase could be overcome in cell free incubations using up to 30 mM methionine in the presence of salts of organic acids such as sodium *p*-toluenesulfonate (*p*TsONa).

(*S*)-Adenosyl-L-methionine (SAM or AdoMet) which is synthesized from methionine and ATP by SAM synthetase (EC 2.5.1.6) plays an important role in many biological reactions.



(-)-AdoMet

SAM contains three transferable groups within its structure. The methyl group is transferred to proteins, lipids, nucleic acids, vitamin B_{12}, *etc.* by SAM-dependent methyltransferases.[1] The 1-amino-1-carboxyl propyl moiety transfers the aminopropyl group to polyamines, spermine and spermidine, following a decarboxylation reaction carried out by AdoMet decarboxylase[2] and is also the precursor of the plant hormone, ethylene.[3] Finally the adenosyl moiety is the precursor of the cyclopentenediol of the tRNA wobble base queuine.[4] SAM also functions as a radical generator like coenzyme-B_{12} in lysine-2,3-aminomutase,[5] ribonucleotide reductase during anaerobic growth,[6] and pyruvate formate-lyase.[7] In the clinical field, SAM has potential importance as a therapeutic drug in the treatment of liver disease and as an anti-depressant.[8]

Genes encoding SAM synthetase have been characterized and sequenced from *E. coli* (*metK* & *metX*),[9] *Saccharomyces cerevisiae* (*sam1* & *sam2*),[10] rat (α, β, & γ forms),[11] mouse,[12] bovine brain,[13] human lymphocytes and liver,[14] *Arabidopsis thaliana* (*sam1* & *sam2*),[15] carnation,[16] and rice.[17] The characteristics of *E. coli* SAM synthetase encoded by *metK* have been described in detail by G. Markham, *et al.*[18] In this enzyme SAM is a competitive inhibitor (K_i = 0.01 mM) against ATP and a noncompetitive inhibitor (K_i = 0.01 mM) against methionine, and the coproducts pyrophosphate (PP_i) and orthophosphate (P_i) are also noncompetitive inhibitors against ATP ($K_{i,PPi}$ = 0.4 mM & $K_{i,Pi}$ = 8 mM) and methionine ($K_{i,PPi}$ = 0.4 mM & $K_{i,Pi}$ = 12 mM).[19] Therefore, the *E. coli* enzyme shows severe product inhibition in incubations containing ≥ 1 mM methionine.[20]

In the present study, we show that this product inhibition could be overcome in cell free incubations using up to 30 mM methionine in the presence of salts of organic acids such as sodium *p*-toluenesulfonate (*p*TsONa).

Product inhibition by SAM may result from either prevention of ATP binding at the ATP binding site[21] or generation of the inactive E-Met-SAM complex formed by random binding of the E-Met complex by SAM.[18] It is not known whether free (cationic) SAM or neutral SAM forming an ion-pair binds SAM synthetase as an inhibiting species. If the cationic form is involved, the SAM binding site of the enzyme can be regarded as a counter anion of the sulfonium ion of SAM. It is suggested that counter anions having a strong binding affinity should compete with the SAM binding site of the enzyme in binding the cationic SAM, which is thus released from the active site during the incubation. However if neutral SAM is involved rather than cationic SAM, the binding ability of SAM synthetase will depend crucially on the size of the ion-paired SAM because enzymes normally have a well defined, but restricted binding-pocket size for the substrate. If a certain concentration of counter anion either having high affinity for SAM or able to enlarge the size of the ion-pair is added to the incubation mixture, the product inhibition effect might decrease or disappear. Matos *et al.* showed that tosylates or sulfates are better counter anions of SAM than halide (I^-, Br^-, & Cl^-) in preventing SAM epimerization.[22] This effect may result from the formation of stronger ion pairs between the sulfonium cation and the tosylate and sulfate anions than with halide anions. Since the tosylate anion is bulky and strong enough to stabilize an ion pair, the conversion of methionine to SAM was investigated by adding different concentrations of *p*TsONa. The effect of sulfate anion was also investigated.

The incubation reactions were easily monitored by ^{13}C-NMR spectroscopy due to the large chemical shift difference of the methyl group in ^{13}C-methyl-methionine (15.2 ppm) and SAM (24.3 ppm) (Fig. 1 A & B). Crude SAM synthetase was prepared from *E. coli* strain DM50 containing plasmid pK8 harboring the SAM synthetase *metK* gene.[23] The DM50(pK8) cells were grown at 37 °C in Luria-Bertani (LB) medium containing 30 μg/ml of either oxytetracycline or tetracycline. About 30 grams of cells were collected by centrifugation and then resuspended in 100 ml of 100 mM Tris buffer (pH 8.0) containing 1 mM EDTA. Lysozyme (50 μg/ml) was added to the solution which was then allowed to stand at room temperature for 30 min. Then phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM.

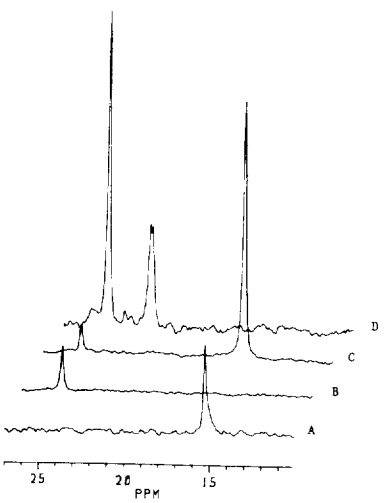


Fig. 1 ^{13}C-NMR Analysis of the enzyme-catalyzed reaction A) ^{13}C-methyl-methionine (^{13}C-Met) in buffer B. B) Reaction containing 1 mM ^{13}C-Met in buffer A. C) Reaction containing 10 mM ^{13}C-Met in buffer B. D) Reaction containing 10 mM methionine and 400 mM *p*TsONa in buffer B. The methyl peak of *p*TsOH appears at 21.9 ppm. 100 mM Tris-HCl was useed as the internal standard. The peak height of 100 mM Tris-HCl was fixed at 5 cm for each spectrum

The cells were lysed by sonication (Model W 200R, Heat Systems-Ultrasonics, Inc., Plainview, NY, 11803) at full power (three times) at < 15 °C and the lysate centrifuged for 20 min at 35,000 rpm in a Beckman Ti 45 rotor to remove cell debris. The supernatant was stored at -20 °C until required.

Complete conversion of 1 mM methionine to SAM occurred (Fig. 1 B) in a mixture of 1.3 mM ATP, 2.6 mM $MgCl_2$, 100 mM KCl, 0.1 % 2-mercaptoethanol (βME), 100 mM Tris-HCl buffer, pH 8.0 (buffer A), and ~4 mg of total protein (300 µl lysate) within 1 h at rt. As previously reported,[20] product inhibition appeared in incubations using 10 mM methionine (Fig. 1 C) in a mixture of 13 mM ATP, 26 mM $MgCl_2$, 100 mM KCl, 0.1 % βME, 100 mM Tris-HCl buffer, pH 8.0 (buffer B), and ~4 mg of total protein (300 µl lysate) at rt. Even when the amount of total protein added to the incubation mixture was increased 7-fold or the incubation time prolonged to 24 h, the product inhibition effect was unchanged (not shown).

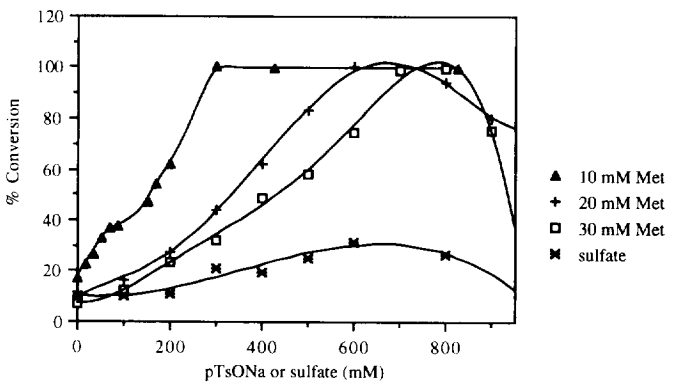


Fig. 2 % Conversion of 10 mM, 20 mM, & 30 mM Met to SAM in the presence of pTsONa or sulfate (at 10 mM Met)

Surprisingly, addition of 400 mM *p*TsONa to the 10 mM scale incubation completely overcome the product inhibition (Fig. 1 D). The alleviatory effect of *p*TsONa was concentration dependent and, indeed, allowed total conversion of substrate within 5 hr at concentrations up to 30 mM methionine in the presence of 1.3 equivalents of ATP and 2.6 equivalents of $MgCl_2$ (Fig. 2). By contrast another isomerization stabilizer, sulfate, did not significantly alleviate the product inhibition (Fig. 2).

The size of the ion-pair may, indeed, be the most important factor in overcoming product inhibition since tosylate and sulfate differ in size but have the same stabilizing effect against SAM epimerization. In the present work, we report on a method for overcoming the problem of product inhibition existing in *E. coli* SAM synthetase, at concentrations up to 30 mM methionine, by adding the bulky counter anion, tosylate, to the cell free system. This result indicates that product inhibition may be overcome by adding appropriate counter ions in other cases similar to that of *E. coli* SAM synthetase. The increasing demand for labeled SAM for the study of SAM-dependent enzyme reactions can now be met, for this study provides an efficient method for the synthesis of labeled SAM using *E. coli* SAM synthetase thanks to the increased availability of the enzyme from genetically engineered *E. coli*,[23] and to the removal of the obstacle of product inhibition. Experiments are in progress to synthesize SAM on a larger scale by this method.

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