

Methionine Adenosyltransferase Purified from Rat Liver

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Received October 31, 2000; revised October 11, 2001; accepted October 12, 2001

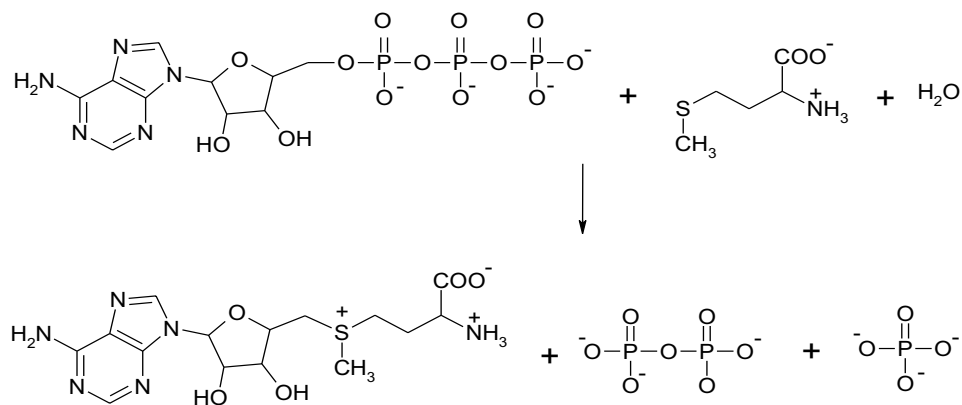
Methionine adenosyltransferase (MAT III), also known as *S*-adenosylmethionine synthetase, was purified from rat liver and crystallized. X-ray diffraction data were collected using a microfocused synchrotron radiation. The crystallization conditions were extensively optimized but final crystal size was never larger than $30^3 \mu\text{m}^3$. Due to their small size crystals had no detectable diffraction on either rotating anode source or the Deresbury SRS beamline 9.6 (GB). Finally, four data sets were collected on Grenoble ESRF (France) undulator microfocus beamline ID13 to resolution of 3.2–3.6 Å. Crystals belong to the cubic space group *F*432 with cell dimension $a = 246 \text{ Å}$. Attempts are under way to solve the structure by molecular replacement, using recombinant MAT I rat liver structure as a search model.

Key words: methionine adenosyltransferase, enzyme, microcrystals, microdiffraction.

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INTRODUCTION

Methionine adenosyltransferase (MAT), also known as *S*-adenosylmethionine synthetase, catalyses the formation of *S*-adenosylmethionine (AdoMet) from L-methionine and ATP (Scheme 1). In mammalian tissues, three forms of MAT have been identified, as products of three genes. Gene MAT 1A generates $\alpha 1$ subunits, MAT 2A generates $\alpha 2$ subunits and MAT 2B generates β subunits.¹⁻⁵ MAT II, specific of extrahepatic tissues and fetal liver, is a heterotetramer formed by two $\alpha 2$ and two β subunits.^{2,6} In the liver, MAT exists as a tetramer (MAT I) and as a dimer (MAT III) of α subunits.^{1,7-11} The two liver enzymes with different kinetic properties, having MAT III the highest metabolic capacity, which enables it to clear the methionine from the diet (a process that takes place in the liver).¹² In the liver, *S*-adenosylmethionine, discovered by Cantoni¹³ in 1953, serves as the methyl donor for many biological reactions and also provides the propylamine group in the synthesis of polyamines. The formation of AdoMet, catalysed by MAT, occurs in a two-step reaction.^{14,15} First, AdoMet is formed by nucleophilic attack of the sulfur atom of methionine on the C5' atom of ATP in an S_N2 reaction, with cleavage of the complete triphosphosphate group from ATP. In the second step, the triphosphosphate chain is hydrolyzed to PPi and Pi before any product is released. MAT requires divalent metal ions such as Mg^{2+} for activity¹³ and is greatly activated by certain monovalent cations as K^+ . The regulatory role of nitric oxide (NO) has also been extensively studied.¹⁶ Comparisons of sequences of MAT from various sources *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, the leaf of *Dianthus caryophyllus*, rat liver, rat kidney, human liver and human kidney show that MAT is an exceptionally well-conserved enzyme through evolution.¹⁷



Scheme 1.

The crystal structure of MAT from *E. coli* was determined at 3 Å resolution.¹⁷ The enzyme consists of 4 identical subunits, each of 383 amino acid residues. Two subunits form a spherical, tight dimer. Each subunit consists of three similar domains that are related to each other by pseudo-3-fold symmetry. The essential divalent and monovalent metal ions lie in a large, deep active site cleft. Recently, the structure of the recombinant rat liver tetrameric MAT I was solved at 2.7 Å in tetragonal space group $P4_122$.¹⁸ The recombinant form of the enzyme purified from inclusion bodies was selected because attempts to crystallize the liver purified protein were unsuccessful.

Our objective is to solve the 3-D structure of dimeric MAT III purified from rat liver by molecular replacement, using the known structure of the tetrameric recombinant rat liver enzyme as a model.

EXPERIMENTAL

Purification

MAT III isozyme was purified as described previously by Cabrero¹ with some minor modifications. The livers of 20 male rats of about 250 g were removed and placed in 300 ml of ice-cold buffer containing 0.3 mol dm⁻³ sucrose, 0.1 mol dm⁻³ EGTA, and 10 mmol dm⁻³ Tris/HCl, pH = 7.4. Just before homogenization, benzamidine, phenylmethylsulfonyl fluoride, and 2-mercaptoethanol were added to final concentrations of 1 mmol dm⁻³, 0.1 mmol dm⁻³, and 0.1% (vol. fraction, ϕ), respectively. The homogenate was clarified by centrifugation at 100 000 × g for 90 min. The supernatant was loaded onto a DEAE-Sepharose Fast Flow column and equilibrated in buffer A (10 mmol dm⁻³ MgSO₄, 1 mmol dm⁻³ EDTA, and 10 mmol dm⁻³ Hepes, pH = 7.5). The enzyme was eluted with a linear gradient of KCl in the same buffer. A single peak of AdoMet synthetase activity eluted at about 220 mmol dm⁻³ KCl. Fractions containing activity were pooled and loaded onto a phenyl-Sepharose equilibrated in buffer A containing 200 mmol dm⁻³ KCl. After washing the column first with equilibration buffer and then with buffer A, MAT III was eluted with buffer A containing 50% (ϕ) dimethyl sulfoxide. The eluate was dialyzed against buffer A and loaded onto a Blue-Sepharose Fast Flow column equilibrated in the same buffer. MAT III eluted in the flowthrough. Fractions containing AdoMet synthetase activity were pooled and concentrated under nitrogen using an Amicon Ultrafiltration cell fitted with a YM30 membrane. The concentrated MAT III solution was cleared from particles and aggregates with a 0.22- μ m filter, and then KCl and dithiothreitol (DTT) were added to give us final concentrations of 10 and 5 mmol dm⁻³, respectively. The final MAT III solution was aliquoted, frozen in liquid nitrogen, and stored at -80 °C. The protein concentration was determined using the Bio-Rad Protein Microassay, with bovine serum albumin as the standard, based on the method of Bradford.¹⁸ The purity of the enzyme, estimated by an overloaded SDS-polyacrylamide gel electrophoresis, was over 95%. About 12 mg of protein were obtained in a typical purification.

Crystallization

Crystals suitable for X-ray diffraction studies were grown using hanging-drop vapour diffusion method (Figure 1) and usually appeared within 4–8 weeks. 1–2 μl of protein in 20 mmol dm^{-3} Hepes buffer pH = 7.0 were mixed with an equal volume reservoir precipitant solution containing 0.4–0.8 mol dm^{-3} $(\text{NH}_4)_2\text{SO}_4$ and 100 mmol dm^{-3} MES buffer (pH = 6.2–6.4) at 19 °C.

In an attempt to obtain an ATP complex, some crystals were soaked in a solution containing 0.8 mol dm^{-3} $(\text{NH}_4)_2\text{SO}_4$, 100 mmol dm^{-3} MES buffer (pH = 6.0) and 10 mmol dm^{-3} ATP for one hour. Similarly, to obtain an NO complex hanging drop containing crystals were exposed to an atmosphere of NO in an hermetic container for 6 days.



Figure 1. Cubic micro-crystals of purified rat liver MAT III in hanging drop.

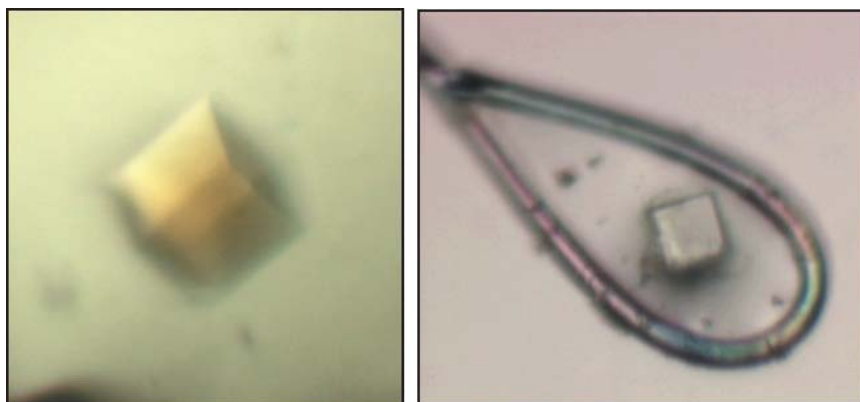


Figure 2. Cubic micro-crystals of purified rat liver MAT III in cryo-loop. Approximate dimensions are $30^3 \mu\text{m}^3$.

The cryo-protectant solution used for X-ray studies contained 0.5 mol dm^{-3} $(\text{NH}_4)_2\text{SO}_4$, 100 mmol dm^{-3} MES buffer (pH = 6.2) and 25% of ethylene glycol. Crystals were soaked in the cryo-protectant solution up to one minute and transferred into the liquid nitrogen (Figure 2). In the case of the ATP soaked crystals the cryo-protectant solution contained 10 mmol dm^{-3} ATP as well.

X-ray Diffraction Experiment

Several data sets were collected on the ESRF ID13 Microfocus beamline²⁰ both from native and ATP-soaked MAT crystals. Data were recorded at 100 K. The crystal-to-detector (MAR CCD 130 mm) distance was 230 mm and oscillation angle was 0.5° . The total oscillation range was 40° to 60° . Images were processed and scaled using DENZO²¹ and Scalepack²² package. Space-group determination was performed using the autoindexing option in DENZO and by inspection of systematic absences among low resolution reflections.

The crystals diffracted up to 3.0 \AA (Figure 3). Details on data processing statistics of two native and two ATP soaked datasets are shown in Table I.

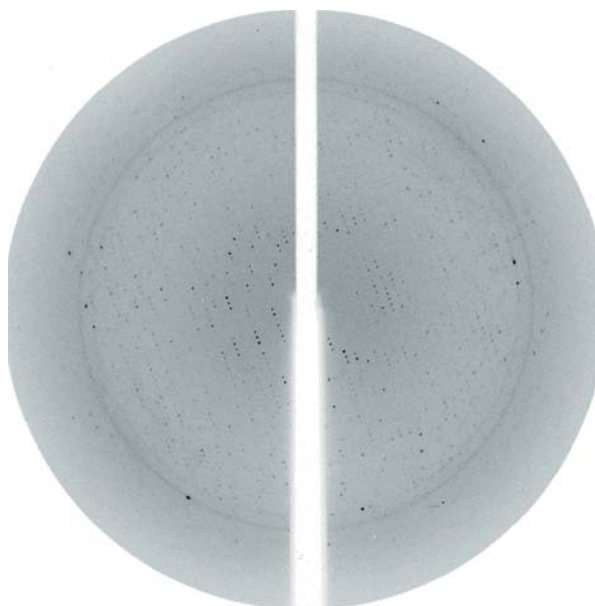


Figure 3. Diffraction pattern from MAT micro-crystal.

RESULTS AND DISCUSSION

Crystallization trials were performed at two different temperatures (4°C and 19°C) using the hanging-drop and the sitting drop vapour-diffusion

TABLE I
Data statistics of the 4 datasets of MAT used

Dataset:	ATP1	ATP3	Nat1	Nat3
No. observed reflexions	126414	124227	145377	18383
No. unique reflexions	10546	7334	7972	9400
$R_{\text{merge}}/\%$	17.8	20.0	21.7	18.6
R_{merge} (highest resol.)/%	63.6	55.7	42.1	48.3
Completeness (all data)/%	94.3	95.6	93.1	89.1
Completeness (highest resol.)/%	97.9	91.5	92.2	90.7
Resolution/Å	3.4	3.2	3.7	3.4
Estimated mosaicity/°	0.39	0.98	1.06	1.08

methods. Crystallization conditions were searched initially by the ammonium sulphate and Hampton crystal screens 1 and 2. The concentration of the protein solution used into screening experiments was 13 mg/ml in 20 mmol dm⁻³ Hepes (pH = 7.0). Having obtained tiny cube-shaped micro-crystals of the enzyme from the solution containing 0.4 to 0.7 mol dm⁻³ (NH₄)₂SO₄, 100 mmol dm⁻³ MES buffer (pH = 5.8) 5 mmol dm⁻³ MgCl₂ and 5 mmol dm⁻³ KH₂PO₄, the optimal conditions for growing crystals for X-ray diffraction study were searched by varying the pH, protein concentration, precipitant, temperature and crystallization. The sitting drop method did not produce crystals while macro-seeding method did not increase crystal size.

Extensive optimization of growth conditions allowed crystal size to be increased, but never larger than 30³ μm³. Due to their small size crystals have no detectable diffraction on either the rotating anode source or the SRS (Daresbury, UK) beamline 9.6. In test experiments at the ESRF (Grenoble, France) beamline ID14 EH2, diffraction spots were recorded up to 5 Å (data not shown), suggesting the cubic space group *F*432 with cell dimension $a = 246$ Å.

Finally, data were collected on ESRF ID13 microfocuss beamline.²³ Four data sets were collected and data collection statistics are given in Table I. Data sets codenamed ATP1 and ATP3 were collected on crystals that were soaked in 10 mmol dm⁻³ ATP solution for an hour while data sets codenamed Nat1 and Nat3 were collected on crystals directly transferred from crystallization drops to cryoprotectant solution. Crystals diffracted to resolution of 3.2–3.6 Å and survived in the microbeam flux for 5 min to allow us the collection of complete data sets for the cubic space group. Diffraction data quality is not high judging from high R_{merge} values shown in Table I. Cry-

stals show variable degrees of mosaicity. MAT crystals soaked in nitric oxide for 6 days did not diffract at all.

Attempts are under way to solve the structure by molecular replacement, using recombinant MAT I rat liver structure as a search model.

Acknowledgements. – We are grateful to the Dr. Frank von Delft and to the staff of ESRF ID13 Microfocus beamline for help with diffraction experiments. We thank Professor Tom Blundell for valuable suggestions and critical reading of the manuscript. The use of EMBO-Grenoble facilities is gratefully acknowledged.

REFERENCES

1. C. Cabrero, J. Puerta, and S. Alemany. *Eur. J. Biochem.* **170** (1987) 299–304.
2. M. Kotb and N. M. Kredich. *J. Biol. Chem.* **260** (1985) 3923–3930.
3. H. L. LeGros, A-B. Halim, A. M. Geller, and M. Kotb. *J. Biol. Chem.* **275** (2000) 2359–2366.
4. G. Okada, H. Teraoka, and K. Tsukada. *Biochemistry* **20** (1981) 934–940.
5. D. M. Sullivan and J. L. Hoffman. *Biochemistry* **22** (1983) 1636–1641.
6. S. Horikawa, J. Sasuga, K. Shimizu, H. Ozasa, and K. Tsukada. *Eur. J. Biochem.* **184** (1989) 497–501.
7. S. Horikawa, M. Ishikawa, H. Ozasa, and K. Tsukada. *J. Biol. Chem.* **265** (1990) 13683–13688.
8. S. Horikawa and K. Tsukada. *Biochem. Int.* **25** (1991) 81–90.
9. L. Alvarez, M. Asuncion, F. Corrales, M. A. Pajares, and J. M. Mato. *FEBS Lett.* **290** (1991) 142–146.
10. L. Alvarez, F. Corrales, A. Martin-Duce, and J. M. Mato. *Biochem. J.* **293** (1993) 481–486.
11. K. Tsukada and G. Okada. *Biochem. Biophys. Res. Commun.* **94** (1980) 1078–1082.
12. S. H. Mudd and J. R. Poole. *Metabolism.* **29** (1975) 707–720.
13. G. L. Cantoni, *J. Biol. Chem.* **204** (1953) 403–416.
14. S. H. Mudd. *J. Biol. Chem.* **237** (1962) PC1372–PC1375.
15. S. H. Mudd. *J. Biol. Chem.* **238** (1963) 2456–2463.
16. I. Perez-Mato, C. Castro, F. A. Ruiz, F. J. Corrales, and J. M. Mato, *J. Biol. Chem.* **274** (1999) 17075–17079.
17. F. Takusagawa, S. Kamitori, S. Misaki, and G. D. Markham, *J. Biol. Chem.* **271** (1996) 136–147.
18. B. Gonzalez, M. A. Pajares, J. A. Hermoso, L. Alvarez, F. Garrido, J. R. Sufrin, and J. Sanz-Aparicio, *J. Mol. Biol.* **00** (2000) 1–13.
19. M. M. Bradford, *Anal. Biochem.* **72** (1976) 248–254.
20. A. Perrakis, F. Cipriani, J. C. Castagna, L. Claustre, M. Burghammer, C. Riekell and S. Cusack, *Acta Crystallogr., Sect. D* **55** (1999) 1765–1770.
21. Z. Otwinowski and W. Minor, *Methods Enzymol.* **276** (1997) 307–326.
22. Collaborative Computational Project, *Acta Crystallogr., Sect. D* **50** (1994) 760–763.
23. P. Engström, S. Fiedler, and C. Riekell, *Rev. Sci. Instrum.* **66** (1995) 1348–1350.

SAŽETAK**Metionin-adenoziltransferaza izolirana iz jetre štakora**

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Enzim metionin-adenoziltransferaza (MAT III), također poznat pod imenom S-adenozilmetioninsintetaza, izoliran je iz jetre štakora i kristaliziran. Kristalografski podatci prikupljeni su upotrebom mikrofokusiranog izvora zračenja na sinkrotronu. Iako su uvjeti kristalizacije bili u velikoj mjeri optimalizirani, konačna veličina kristala nije prelazila $30^3 \mu\text{m}^3$. Zbog malog volumena kristala nije detektirana difrakcija rentgenskog zračenja iz rentgenske cijevi s rotirajućom anodom, kao ni na stanici 9.6 sinkrotrona SRS (Daresbury, Velika Britanija). Četiri skupa podataka prikupljena su do rezolucije 3.2–3.6 Å na stanici ID13 mikrofokusiranim zračenjem undulatora na sinkrotronu ESRF (Grenoble, Francuska). Kristali pripadaju kubičnoj prostornoj grupi $F432$, s dimenzijama ćelije $a = 246 \text{ Å}$. U tijeku je rješavanje kristalne strukture metodom molekulske zamjene, pri čemu se kao polazni model koristi struktura rekombinantne MAT I iz jetre štakora.