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PROPERTIES OF A METHIONYL-tRNA SYNTHETASE FROM *SARCINA LUTEA**

GERALD A. HAHN** AND JOHN W. BROWN

Department of Biochemistry, University of Louisville, School of Medicine, Louisville, Ky. (U.S.A.)

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SUMMARY

Methionyl-tRNA synthetase was purified from sonic extracts of *Sarcina lutea* by successive elution from calcium phosphate gel followed by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. When methionine activation was measured by hydroxamate formation, ATP–$^{32}PP_i$ exchange and attachment to tRNA, the ratio of activities determined by these methods remained constant throughout the purification. The kinetics of the enzymatic reaction were determined using all three assays.

The reaction was inhibited by various salts at high ionic strength, certain nucleotides and RNA from several sources. Reduced glutathione and mercaptoethanol also were inhibitory. Selenomethionine and methionine analogues with alkyl-substituted thiol groups stimulated both ATP–$^{32}PP_i$ exchange and hydroxamate formation. Of the other methionine analogues tested, homocysteine was the most potent inhibitor.

INTRODUCTION

The purpose of this investigation was to study the mechanism of amino acid activation by determining those factors which influence the formation and stability of the aminoacyl adenylate. L-Methionyl-tRNA synthetase (EC 6.1.1.10) was chosen because of its stability and high activity in crude bacterial extracts. The ability of amino acid analogues to serve as substrates or to act as inhibitors of aminoacyl adenylate formation was studied to establish which groups of the amino acid were necessary for binding to the enzyme. To provide further insight into the mechanism of amino acid activation, the effects of added RNA, sulfhydryl reagents, nucleotides and various metal cations on aminoacyl adenylate formation also were investigated.

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** Present address: Department of Pharmacology, The George Washington University School of Medicine, Washington, D.C. 20 005, U.S.A.

EXPERIMENTAL PROCEDURES

Assay for synthetase activity

The determination of synthetase activity by ATP–$^{32}PP_i$ exchange and hydroxamic acid formation has been described previously[1]. The method of MOUSTAFA[2] was used for the measurement of methionine attachment to tRNA. Each reaction vessel contained 100 μmoles Tris (pH 8.0), 10 μmoles $MgCl_2$, 10 μmoles [^{14}C]methionine (Schwartz Biochemicals, 277 500 counts/min), 5 μmoles ATP, 50 μg *Sarcina lutea* tRNA and a limiting amount of enzyme in a final volume of 1.0 ml. Duplicate samples were counted in a Nuclear-Chicago D-47 thin-window, gas-flow counter with a ^{14}C efficiency of 25%.

RNA was prepared by the method of HOLLEY *et al.*[3] and determined quantitatively by the method of SCHNEIDER[4] using yeast high-molecular-weight RNA (Worthington Biochemical Corp.) as the standard.

1 munit of synthetase is defined as that amount of enzyme which will catalyze the transformation of 1 mμmole of substrate per min at 37° under the conditions of the assay procedure.

Preparation of methionyl-tRNA synthetase

Methods described previously[1] were used to grow *S. lutea* and to obtain cell-free extracts. All of the steps in the preparation of the enzyme were carried out at 0–4°. The ATP–$^{32}PP_i$ exchange assay was used routinely to follow purification.

A sonic extract of *S. lutea* prepared in 0.01 M potassium phosphate buffer (pH 6.7) was stirred intermittently for 10 min with calcium phosphate gel prepared by the method of SINGER AND KEARNEY[5]. The ratio of dry weight of calcium phosphate gel to protein generally was 4:1. After centrifugation at 3000 $\times$ *g* for 2 min, the supernatant solution was discarded. Methionyl-tRNA synthetase was eluted from the gel using increasing concentrations of potassium phosphate buffer at pH 6.7. The first eluate in 0.015 M buffer was discarded. Most of the activity was removed from the gel by increasing the concentration of the buffer to 0.03 M. (Incremental increases in the buffer concentration up to 0.06 M resulted in complete elution of the activity).

Solid $(NH_4)_2SO_4$ was added step-wise to the eluate in 0.03 M buffer. Precipitates obtained at 35, 40, 50, and 60% $(NH_4)_2SO_4$ saturation were collected by centrifugation at 20 000 $\times$ *g* for 10 min. The pellets were dissolved in 0.01 M phosphate buffer at pH 6.7 and dialyzed overnight against 100 vol. of this buffer. The fraction obtained by increasing the concentration of $(NH_4)_2SO_4$ from 40 to 50% had a specific activity three times as great as the calcium phosphate gel eluate. Other fractions yielded less than a two-fold purification.

DEAE-cellulose washed by the method of PETERSON AND SOBER[6] was equilibrated with 0.01 M potassium phosphate buffer (pH 6.7) containing 0.3 M KCl and packed in a 1.2 cm $\times$ 36 cm column. After the sample was transfered to the column, it was washed with 50 ml of the buffer before initiating a continuous linear gradient of KCl up to 0.6 M. Approx. 60 fractions of 5 ml each were collected, and portions of each tube were assayed directly.

The highest methionyl-tRNA synthetase activity was found in Fractions 35–37. Further analyses demonstrated that these fractions contained less than 1% contami-

TABLE I

PURIFICATION OF METHIONYL-tRNA SYNTHETASE

(a) Each reaction vessel contained in 1 ml: 100 μmoles Tris (pH 8.0), 10 μmoles of L-methionine, 5 μmoles ATP, 10 μmoles $MgCl_2$, 5 μmoles $^{32}PP_i$, 10 μmoles of KF and a limiting amount of enzyme. (b) Each reaction vessel contained in 3 ml: 3000 μmoles NH_2OH–HCl, 30 μmoles $MgCl_2$, 30 μmoles Tris (pH 8.0), 15 μmoles ATP, 30 μmoles of L-methionine and a limiting amount of enzyme. (c) Each reaction vessel contained in 1 ml: 100 μmoles Tris (pH 8.0), 10 μmoles $MgCl_2$, 10 μmoles [^{14}C]methionine, 5 μmoles ATP, 5 μg *S. lutea* tRNA preparation and a limiting amount of enzyme.

Fraction	*Specific activity (munits/mg protein)*			*Yield (%)*	*Average relative specific activity*
	(a) ATP–$^{32}PP_i$ exchange	*(b) Hydroxamic acid*	*(c) Attachment to tRNA*		
Sonic extract	24	0.92	0.25	(100)	(1)
Calcium phosphate gel 0.03 M phosphate eluate	262	9.67	2.67	47	11
$(NH_4)_2SO_4$ precipitate 40–50% saturation	826	30.5	8.42	21	34
DEAE-cellulose, Fractions 35–37	3650	135	37.2	14	149

nation with isoleucyl- and cysteinyl-tRNA synthetases, and no other synthetase activity was detected. Rechromatography of Fractions 35–37 on Ecteola-cellulose did not increase the specific activity of the preparation.

The activity of the preparation at various stages of purification as measured by all three assay systems is presented in Table I. Evidence that the three assays measured the same enzyme is demonstrated by the fact that the ratio of the rates remained constant at about 100:4:1 at all stages of purification. The purified enzyme in Fractions 35–37 was used in all of the experiments described below.

Materials

α-Methyl-DL-methionine, α-aminobutyric acid, β-aminobutyric acid, γ-aminobutyric acid, L-homoserine, D- and L-ethionine, D-methionine, L-methionine sulfone and DL-cystathionine were purchased from Cyclo Chemical Corp. DL-Methionine sulfoximine, DL-homocysteine, L-methionine, L-norleucine, *N*-acetyl-DL-methionine, α-hydroxymethionine, reduced glutathione, EDTA, 2-mercaptoethanol, *p*-mercuribenzoate and sodium salts of AMP, GMP, CMP, UMP, ATP, GTP, CTP, UTP, were obtained from Nutritional Biochemicals Corp. DL-Selenomethionine and DL-methionine amide were gifts of Dr. K. P. McCONNELL.

RESULTS AND DISCUSSION

General properties

K_m values relative to methionine, ATP and PP_i were determined by all three assays for methionyl-tRNA synthetase activity. The values shown in Table II were calculated from Lineweaver–Burk plots. The K_m value of 0.58 mM for methionine in the exchange reaction is about 25 times that obtained by BERGMANN, BERG AND DIECKMANN[7] under comparable conditions using a preparation of the enzyme from *Escherichia coli*.

TABLE II

METHIONYL-tRNA SYNTHETASE K_m VALUES OBTAINED BY VARIOUS ASSAYS

Conditions for each assay were the same as stated in Table I. K_m values were calculated from Lineweaver–Burk plots.

Assay procedure	*K_m relative to substrate*		
	Methionine (mM)	*ATP (mM)*	*PP_i (mM)*
Hydroxamic acid formation	1.9	2.7	
ATP-$^{32}PP_i$ exchange	0.58	1.3	0.25
Attachment to tRNA	2.0	2.2	

The pH optimum for the synthetase from *S. lutea* was broad with a maximum in the range 7.8–8.2. Enzyme activities at pH 7.0 and 9.0 were 70 and 85% of the maximum, respectively.

ATP was present in all assays at its optimal concentration, 5 mM. CTP, GTP and UTP at this concentration did not replace ATP. Concentrations of ATP and PP_i greater than 5 mM inhibited activity as measured by the exchange reaction. Inhibition by PP_i may have been due to binding of Mg^{2+} as suggested by HEPPEL AND HILMOE[8].

The common nucleoside 5'-monophosphates acted as competitive inhibitors of the synthetase reaction. As anticipated, AMP was the most active. The K_i values

TABLE III

EFFECT OF DIVALENT CATIONS AND EDTA ON METHIONINE ACTIVATION

Conditions for each assay were the same as stated in Table I. Additions were made to the complete system without $MgCl_2$. EDTA was preincubated with the enzyme for 10 min at 0° prior to its addition to the reaction vessel.

Additions	*ATP–$^{32}PP_i$ exchange (munits)*	*Hydroxamic acid formation (munits)*	*Attachment to tRNA (munits)*
No addition	0.8	0.22	0.04
$MgCl_2$ (10 mM)	21.5	9.31	0.88
$MnCl_2$ (10 mM)	6.0	1.53	0.21
$CoCl_2$ (10 mM)	7.6	2.14	0.39
EDTA (10 mM)	0.1	0.03	0.02

for AMP, GMP, CMP and UMP related to methionine as substrate in the exchange reaction were 0.47, 2.23, 14.3 and 48.3 mM, respectively. Presumably, the mechanism of this inhibition resides in the ability of these nucleotides to compete with (labeled) PP_i in the nucleophilic displacement reaction with enzyme-bound aminoacyl adenylate, the reverse of the amino acid-activation reaction.

Effects of metal ions

In general Mg^{2+}, Co^{2+} or Mn^{2+} are required for aminoacyl-tRNA synthetase activity[9]. The evidence presented in Table III demonstrates that these ions at a

10 mM concentration also will stimulate activity of the methionyl-tRNA synthetase under investigation. Ni^{2+}, Ba^{2+}, Fe^{2+}, Ca^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} also were tested, but none of these ions stimulated activity of this enzyme. EDTA presumably inhibited activity by binding metal cations associated with the enzyme.

HOLLEY *et al.*[3] observed that Na^+, NH_4^+, Li^+ and K^+ inhibited the ATP–$^{32}PP_i$ exchange reaction catalyzed by a tyrosyl-tRNA synthetase. Others have noted a stimulation of synthetase activity by monovalent cations[10]. As shown in Table IV,

TABLE IV

ACTIVITY OF METHIONYL-tRNA SYNTHETASE IN THE PRESENCE OF VARIOUS MONOVALENT CATIONS

Conditions for each assay were as stated in Table I (a). All cations were added as their chloride salts.

Cation added to complete system	*Concn. of cation added*	*ATP–$^{32}PP_i$ exchange (munits)**	
		10 mM	*100 mM*
NH_4^+		31.6	28.1
Na^+		32.7	15.1
K^+		37.1	31.2

* A value of 34.5 munits was obtained when no monovalent cation was added.

the methionyl-tRNA synthetase from *S. lutea* appears to be stimulated by 10 mM KCl and inhibited by 100 mM NaCl. At sufficiently high concentrations all acted as competitive inhibitors, and on the basis of the exchange reaction the K_i values for NaCl, NH_4Cl, LiCl and KCl were 21, 59, 80 and 159 mM, respectively.

Effects of sulfhydryl reagents

STERN *et al.*[11] reported that all of the synthetases from *E. coli* except lysyl-tRNA synthetase were inhibited by *p*-mercuribenzoate. The results of our studies of the synthetases from *S. lutea* support this finding in general with the exception of those enzymes which catalyze the activation of the sulfur amino acids, cysteine and methionine[1]. When purified methionyl-tRNA synthetase from *S. lutea* was tested, it was found that 1 mM *p*-mercuribenzoate diminished the ATP–$^{32}PP_i$ exchange rate by only 7%.

On this basis it might be presumed that sulfhydryl compounds such as reduced glutathione or 2-mercaptoethanol would have little effect upon methionine activation. As shown in Table V, the presumption is not quite correct. Perhaps the inhibition noted is due to a nucleophilic displacement reaction between enzyme-bound aminoacyl adenylate and the sulfhydryl reagents.

Inhibition by RNA

RNA from four sources was tested for its effect on the exchange reaction. All four resulted in a non-competitive inhibition with respect to methionine as substrate. The K_i values for RNA preparations from *S. lutea*, yeast, *E. coli* and beef liver were

TABLE V

EFFECT OF SULFHYDRYL COMPOUNDS ON METHIONINE-DEPENDENT ATP–$^{32}PP_i$ EXCHANGE

Additions were made to the complete assay system as stated in Table I (a).

Addition	*Concentration (mM)*	*Activity (munits)*
None		10.7
Reduced glutathione	1	7.7
Reduced glutathione	10	6.5
2-Mercaptoethanol	1	7.4
2-Mercaptoethanol	10	6.1

calculated to be 2.2, 3.6, 3.8 and 4.7 μg/ml, respectively. The effectiveness of each RNA as an inhibitor could not be correlated with its acceptor capacity for activated methionine. Possibly inhibition was due to binding of the RNA at an allosteric site which resulted in a conformational change, decreasing the stability of the complex between enzyme and the aminoacyl adenylate.

Effect of methionine analogues

NISMANN AND HIRSH[12] observed the activation of methionine, selenomethionine, ethionine and norleucine with crude extracts of *E. coli*. BERG[13] reported the activation of methionine and ethionine using a partially purified methionyl-tRNA synthetase from brewers yeast. Methionine sulfoxide, methionine sulfone and homocystine were not activated. As shown in Table VI, methionine, ethionine, cystathionine and selenomethionine all stimulated ATP–$^{32}PP_i$ exchange catalyzed by the enzyme from *S. lutea*. K_m and V values obtained using selenomethionine were identical with those obtained using methionine. When ethionine and cystathionine were tested as substrates, the K_m values were increased approx. 5 times, indicating that lengthening of the *S*-alkyl substituent diminished the ability of these compounds to form an ES complex.

In the hydroxamic acid assay, methionine, ethionine and cystathionine were found to be capable of forming hydroxamic acids. Selenomethionine was not tested in this assay. D-Methionine and D-ethionine were inactive at concentrations 10 times that used in the standard assay system.

TABLE VI

STIMULATION OF ATP–$^{32}PP_i$ EXCHANGE BY ANALOGUES OF METHIONINE

Conditions for each assay were as stated in Table I (a), except that varying concentrations of analogue replaced methionine in all but the control system.

Substrate	K_m* *(mM)*	V *(%)*
L-Methionine (control)	0.57	(100)
DL-Selenomethionine	0.57	100
L-Ethionine	2.94	93
DL-Cystathionine	3.22	87

* K_m values were calculated from Lineweaver–Burk plots in each case for the L-isomer only.

Homoserine, α-aminobutyric acid, β-aminobutyric, γ-aminobutyric acid, α-methylmethionine, α-hydroxymethionine, methionine sulfone, methionine sulfoximine, methionine amide, *N*-acetylmethionine, norleucine and homocysteine were inactive in the ATP–$^{32}PP_i$ exchange assay and were tested for their ability to serve as inhibitors of this exchange with respect to methionine. These data are summarized in Table VII. D-Methionine and D-ethionine did not inhibit even at a concentration of 100 mM, 10 times the concentration tested in the standard assay. Methionine analogues containing substituents on the carboxyl or amino groups act as non-competitive inhibitors. All others inhibited competitively.

TABLE VII

INHIBITION OF METHIONINE-DEPENDENT ATP–$^{32}PP_i$ EXCHANGE BY METHIONINE ANALOGUES

Lineweaver–Burk plots were used to determine K_i values. The concentration of the L-isomer only was used in the calculations. Conditions for each assay were as stated in Table I (a), except that the concentration of methionine was varied in the presence of a constant concentration of each analogue. K_i values shown are the averages from two series of experiments.

Analogue	*Concentration of L-isomer tested (mM)*	*Type of inhibition*	K_i *(mM)*
Methionine amide	10, 20	Non-competitive	96.6
N-Acetylmethionine	10, 20	Non-competitive	13.1
α-Aminobutyric acid	10, 100	Competitive	12.5
β-Aminobutyric acid	10	Competitive	11.7
γ-Aminobutyric acid	10, 100	Competitive	104
α-Methylmethionine	10, 100	Competitive	8.6
α-Hydroxymethionine	10	Competitive	8.8
Homocysteine	10	Competitive	3.1
Homoserine	10	Competitive	8.6
Norleucine	20	Competitive	100
Methionine sulfone	10	Competitive	15.1
Methionine sulfoximine	10, 100	Competitive	16.5

Homocysteine was the most effective competitive inhibitor tested. Homoserine, the O-analogue of homocysteine, also was quite inhibitory. The least effective inhibitors were norleucine and γ-aminobutyric acid, analogues in which sulfur is replaced by $-CH_2-$ or $-NH_2$ groups. From these data it appears that a sulfur (or oxygen) substituent on the γ-position is necessary for enzyme complex formation. However, this would not explain the inhibitory effect of α- and β-aminobutyric acids, which have no functional substituent on the γ-position. Neither would it explain the activity of the α-substituted methionines. Therefore, we presume that binding to the enzyme is related to both the sulfur group (whether substituted or not) and substituents on the butyryl moiety of methionine.

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