TWO FORMS OF METHIONYL-TRANSFER RNA SYNTHETASE FROM MYCOBACTERIUM SMEGMATIS

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SUMMARY: Two methionyl-transfer RNA synthetases (A and B forms) have been isolated from Mycobacterium smegmatis. The homogeneous preparations of the enzymes showed 1500 fold increase in specific activity in aminoacylation of methionine specific tRNA. The A and B forms differed in their specificity of aminoacylation of $tRNA_m^{Met}$ and $tRNA_f^{Met}$; enzyme B exhibited much higher specificity for $tRNA_f^{Met}$. The molecular activities of A and B enzymes for aminoacid and tRNA were identical. The turnover number for aminoacid was 27 fold greater than that for tRNA, while the Km values for tRNA were lower by a factor of 10^6 as compared to the aminoacid. Both the enzymes catalysed ATP-pyrophosphate exchange reaction to the same extent.

Aminoacyl-tRNA synthetases catalyse the esterification of aminoacids to tRNA in a highly specific manner and thereby ensure the correct translation of the genetic code. Occurrence of different isoacceptor tRNAs at various stages of the organismal growth may in fact suggest the preferential aminoacylation of a particular species of tRNA at a defined physiological condition. The differential use of isoaccepting species of tRNA at a given stage of growth or development is only a function of messenger sequence, since the synthetases do not distinguish between isospecies of cognate tRNA. The presence of more than one aminoacyl-tRNA synthetase for a single aminoacid demonstrated in a few cases have all been attributed to proteolytic cleavage or subunit separation during enzyme isolation or as a result of phage induced modification [1-3].

The distinction between the different isoacceptor species of tRNA may become a desirable step in the regulation, especially in cases like met- and formylmet-specific tRNAs. The two species of methionine specific tRNA have different functions, viz., to recognise either the initiator codon or the internal methionine codons; but so far, there are no reports on the multiplicity of methionyl-tRNA synthetase. In this communication, we report the presence of two forms of methionyl-tRNA synthetase (EC 6.1.1.10) from Mycobacterium smegmatis. These two forms of enzymes show different specificities for $tRNA_m^{Met}$ and $tRNA_f^{Met}$ in aminoacylation reaction.

MATERIALS AND METHODS

Buffers: A. 0.01M Tris-HCl (pH 7.4), 0.02M 2-Mercaptoethanol, 0.01M $MgCl_2$ and 10% Glycerol. B. 0.02M Potassium phosphate (pH 7.6), 0.02M 2-Mercaptoethanol, 0.005M $MgCl_2$ and 10% Glycerol. C. 0.25M Potassium phosphate (pH 6.5), 0.02M 2-Mercaptoethanol, 0.005M $MgCl_2$ and 10% Glycerol. D. 0.02M Potassium phosphate (pH 6.5), 0.02M 2-Mercaptoethanol, 0.005M $MgCl_2$ and 10% Glycerol.

Isolation and Purification of Methionyl-tRNA Synthetase: M. smegmatis cells grown at 37° as submerged culture in a synthetic medium [4], were harvested in the late exponential growth phase (36 hrs). The washed cells were ground with twice the amount of Alumina and suspended in buffer A. During large scale preparations, the cells suspended in buffer A (2 ml/gm wet weight of cells) were subjected to sonic disintegration for 20 min in Raytheon sonic oscillator (10 Kc/sec), or for 5 min in MSE 150W ultrasonic disintegrator. The S-25 and S-100 supernatants were prepared by centrifugation at 25,000xg for 30 min and 100,000xg for 90 min respectively. The 100,000xg supernatant (S-100) was loaded on a DEAE-cellulose column (25 cm x 2 cm) previously equilibrated with buffer B. The absorbed proteins were eluted in a batchwise fashion using buffer C (higher molarity of potassium phosphate and lower pH than buffer B), followed by buffer C containing 0.4M NaCl. The protein fractions showing methionyl-tRNA synthetase activity (eluted in presence of 0.4M NaCl) were pooled and fractionated by precipitation with ammonium sulfate. The fraction precipitating between 45-80% saturation of ammonium sulfate was dissolved in buffer D, dialysed against the same buffer and fractionated on a DEAE-cellulose column. The enzyme activity was distributed into two distinct peaks (Fig. 1, peaks A and B); the fractions showing maximum activity from each peak were pooled separately. The pooled fractions were dialysed against buffer D and concentrated subsequently by dialysis against buffer D containing 7% Aquacide-I.

The concentrated protein peaks A and B were separately passed through a Sephadex G-200 column (60 x 1.5 cm) and the peak fractions were pooled, concentrated as described earlier and refiltered through Sephadex G-200 column.

Protein was estimated using Lowry's method as modified by Hartree [5].

Polyacrylamide gels at pH 8.7 and pH 4.5 were prepared and run according to the methods of Ornstein [6] and Davis [7]. Sodium dodecylsulfate polyacrylamide gel electrophoresis was run according to the methods of Weber and Osborn [8].

Transfer RNA was isolated from M. smegmatis using phenol extraction method [9].

The aminoacid acceptor assay system consisted of 0.1M cacodylate buffer (pH 6.5), 10 mM magnesium acetate, 2 mM ATP (pH 7.0), 10 mM KCl, 60 µg (1.5 $A_{260\ nm}$ units) of total tRNA (unless specifically mentioned), L-[^{3}H]methionine (25,000 cpm, sp. activity 290 mCi/mmole) and enzyme in a final volume of 0.125 ml. After incubation at 35° for 20 min, 0.1 ml of reaction mixture was layered on Whatman 3MM filter paper squares (1.8 cm x 1.8 cm) and the aminoacyl-tRNA formed was estimated using a Beckman LS-100 scintillation spectrometer.

One unit of enzyme activity is defined as the amount of enzyme that incorporates 1 µmole of [^{3}H]methionine into tRNA in 20 min at 35° under above conditions.

ATP-PP_i exchange assays were carried out as described by Calender and Berg [10].

One unit of enzyme activity is defined as that amount of enzyme which forms 1 µmole of [^{32}P]ATP in 15 min at 35°.

RESULTS

Purification of methionyl-tRNA synthetase: During purification, at the stage of DEAE-cellulose chromatography the enzyme was resolved into two peaks of activity (Fig. 1, peaks A and B). The fractions showing maximal activity were tested using total tRNA from E. coli and M. smegmatis as well as pure $tRNA_f^{Met}$ and $tRNA_m^{Met}$ from E. coli as substrates. The 'A' fraction showed no distinction between the different substrates in the extent of aminoacylation whereas the 'B' fraction showed much higher activity with $tRNA_f^{Met}$.

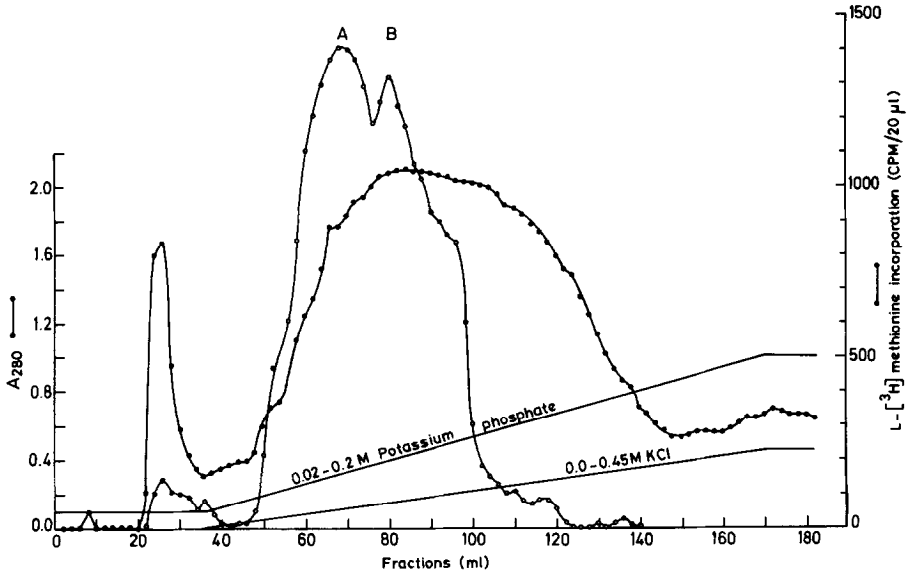

Fig. 1. DEAE-cellulose elution profile of methionyl-tRNA synthetases.

The ammonium sulfate fraction (45-80% saturation) containing enzyme activity was loaded on 25 x 1.5 cm DEAE-cellulose column equilibrated with buffer D. Simultaneous linear gradients of 0.02M-0.25M potassium phosphate (pH 6.5) and 0-0.45M KCl were used to elute the proteins. Fractions were assayed using $tRNA_f^{Met}$ from E. coli as substrate.

●——● A_{280} nm

○——○ L-[^{3}H]methionine incorporation.

The separated 'A' and 'B' fractions when passed through Sephadex G-200, were located at different volumes of buffer (Fig. 2 and 3). The peak B fractions, eluting later from the DEAE-cellulose column, appeared earlier than 'A' on gel-filtration. The difference of distribution of 'A' and 'B' fractions was confirmed by using larger bed volume of G-200 for gel filtration as well as by studying the distribution of a marker enzyme (valyl-tRNA synthetase from M. smegmatis) under identical conditions. The fractions showing

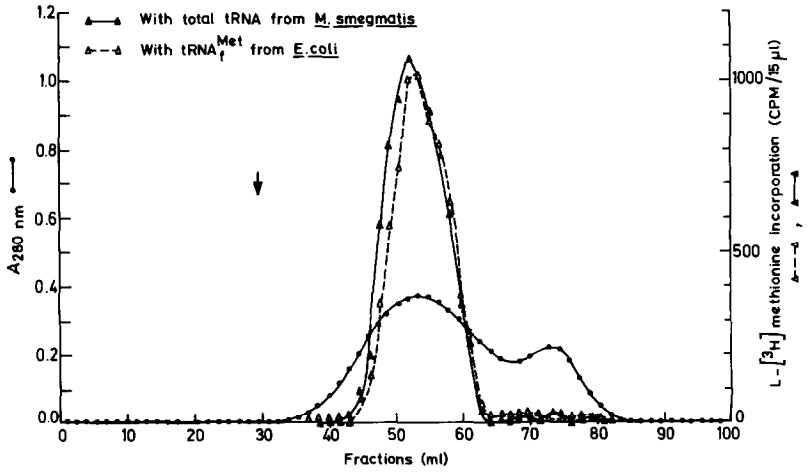

Fig. 2. Sephadex G-200 filtration of DEAE-cellulose 'A' fraction.

Concentrated fraction 'A' (from 66 ml to 72 ml, eluted from the DEAE-cellulose column shown in Fig. 1) was gel filtered through a Sephadex G-200 column (60 x 1.5 cm). The flow rate was 4.5 ml per hour.

●——● A_{280} nm

▲——▲ L-[^{3}H]methionine incorporation with total tRNA from M. smegmatis.

△- - - -△ L-[^{3}H]methionine incorporation with tRNA$_f^{Met}$ from E. coli.

Arrow indicates void volume.

the maximum activity when pooled and passed again through the column of Sephadex G-200 gave a single coincident peak of protein and enzyme activity (Fig. 3, inset). The summary of the purification procedure is presented in Table 1. Both enzymes A and B, on polyacrylamide gel electrophoresis showed the presence of a single band at alkaline or acidic pH. The 'A' and 'B' enzymes did not show any significant difference in mobilities on 7.5% polyacrylamide gel and behaved like highly negatively-charged polypeptides.

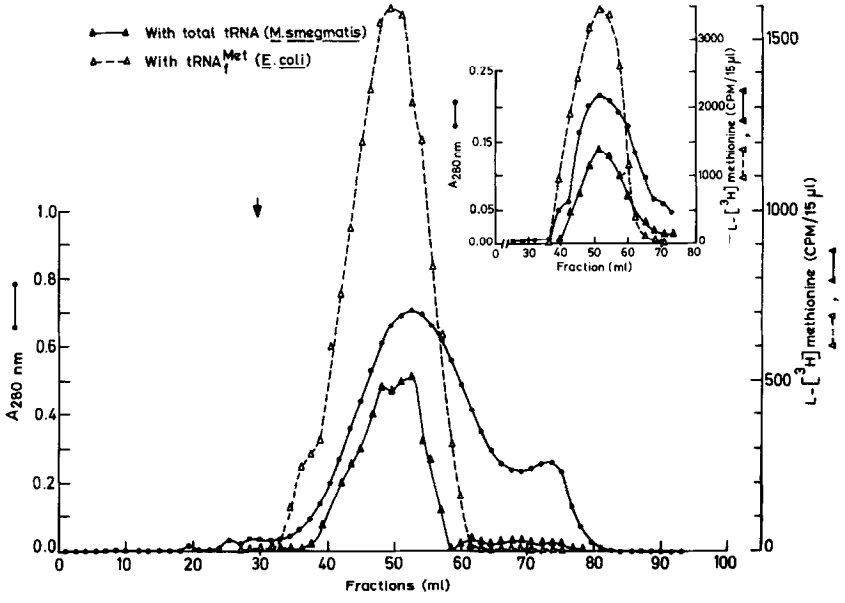

Fig. 3. Sephadex G-200 filtration of DEAE-cellulose 'B' fraction.

Fraction 'B' (from 78 to 82 ml, eluted from DEAE-cellulose column shown in Fig. 1) was gel filtered through a Sephadex G-200 column. Conditions were same as under Fig. 2. Peak activity (51 ml to 54 ml) was pooled, concentrated and refiltered through the same column (Fig. 3, inset) under identical conditions.

●——● $A_{280\ nm}$

▲——▲ L-[^{3}H]methionine incorporation with total tRNA from M. smegmatis.

△- - - -△ L-[^{3}H]methionine incorporation with $tRNA_f^{Met}$ from E. coli.

Molecular weight determination: The molecular weights of the enzymes were determined by gel-filtration on a calibrated column of Sephadex G-200 and the values obtained were 65,000 and 85,000 daltons for A and B enzyme preparations respectively (Fig. 4a). Thus, the A and B enzymes seem to differ in their molecular weights by 20,000 daltons.

When the enzymes were subjected to polyacrylamide gel electro-

TABLE 1: Purification of methionyl-tRNA synthetase

Fraction	Total protein (mg)	Specific activity*	Fold purification	% Yield
S-25	3900	1.1	1	
S-100	2900	7.8	7.3	100
Ammonium sulfate (45-80%)	240	54.9	51	57.9
DEAE-cellulose II				
A	18.7	545.2	510	44.8
B	22.0	406.5	369.5	39.5
Sephadex G-200				
A	2.5	1880.0 ($tRNA_f^{Met}$)	1760	20.7
		1800.0 (total tRNA)	1685	19.8
B	5.0	1600.0 ($tRNA_f^{Met}$)	1500	35.2
		800.0 (total tRNA)		

* nmoles of L-[^{3}H]methionine incorporated in 20 min per mg protein.

phoresis in presence of SDS, much lower values were obtained for their molecular weights (Fig. 4b). The enzyme A gave a prominent band, corresponding to molecular weight of 31,000 and a weak band of 65,000 (corresponding to the undissociated enzyme). The B enzyme behaved similar to the A enzyme but for the appearance of an extra

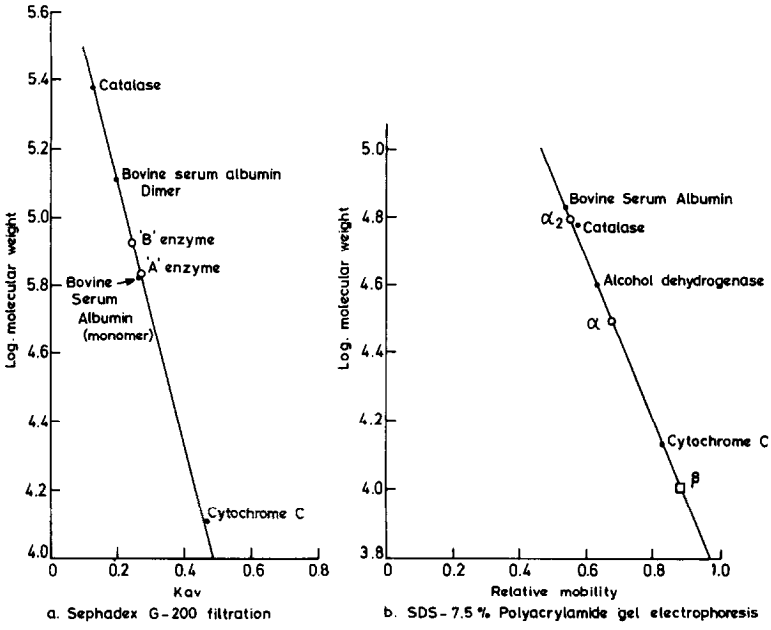

Fig. 4. Determination of molecular weights of methionyl-tRNA synthetases 'A' and 'B'.

4a. By gel filtration on Sephadex G-200.

Catalase, bovine serum albumin and cytochrome c were used as marker proteins.

4b. By electrophoresis in 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate.

Only the peak fractions after refiltration of the enzyme on Sephadex G-200 were used for electrophoretic studies.

Marker proteins, viz., bovine serum albumin, catalase, yeast alcohol dehydrogenase and cytochrome c, were used. A weak band corresponding to 65,000 daltons (undissociated enzyme A) has also been denoted in this figure. The band corresponding to β was obtained only from enzyme B.

protein band corresponding to a molecular weight of 10,000.

Differential recognitions by enzymes A and B: The enzymes A and B showed different extents of aminoacylation when total tRNA from E. coli (data not shown) or M. smegmatis and pure $tRNA_f^{Met}$

from E. coli were used as substrates (Fig. 2 and 3). Although the specific activities of the enzymes were comparable when $tRNA_f^{Met}$ was used, enzyme B showed much lower activity (40%) with total tRNA as substrate indicating its specificity towards $tRNA_f^{Met}$. This amount of activity agreed well with the content of $tRNA_f^{Met}$ in total methionine specific tRNA [11].

In order to confirm the specificity of B enzyme, pure preparations of $tRNA_m^{Met}$ and $tRNA_f^{Met}$ from E. coli were also used as substrates. Whereas the enzyme A showed equal activity with both the substrates, enzyme B showed only 35% of aminoacylation with $tRNA_m^{Met}$ as compared to $tRNA_f^{Met}$ as substrate. The results were also verified in homologous system. Here again, enzyme B showed higher activities with the formylatable species; in contrast, the extent of aminoacylation was equal with both the species, when enzyme A was employed.

Other properties of the methionyl-tRNA synthetase: The enzyme activity was found to be absolutely dependent on Mg^{2+}; 10 mM Mg^{2+} was the optimal concentration. Both the enzymes A and B exhibited maximum aminoacylation activity at pH 6.5 and at 35°. The activity was similar in sodium cacodylate or Tris buffer.

The enzyme showed a high degree of specificity without any cross reactivity of mischarging. None of the other 18 aminoacids were activated by the enzyme in ATP-PP_i exchange reaction. In aminoacylation reaction also no other labelled aminoacid than methionine was incorporated when total tRNA from M. smegmatis and and E. coli or $tRNA_m^{Met}$ or $tRNA_f^{Met}$ from E. coli were used as substrates. The ATP-PP_i exchange reaction catalysed by this enzyme was absolutely independent of tRNA.

TABLE 2: Kinetic properties of methionyl-tRNA synthetase.

Substrate	Enzyme	Km (M)	V (M)	Molecular activity*
$tRNA_m^{Met}$	A	1.56×10^{-10}	7.56×10^{-10}	
$tRNA_f^{Met}$	A	1.49×10^{-10}	11.11×10^{-10}	6.95
$tRNA_m^{Met}$	B	58.18×10^{-10}	14.00×10^{-10}	
$tRNA_f^{Met}$	B	0.17×10^{-10}	11.10×10^{-10}	6.80
$tRNA_{(total)}^{Met}$	A			6.75
$tRNA_{(total)}^{Met}$	B			3.40
L-Methionine**	A	1.66×10^{-4}	2.85×10^{-6}	176.50
L-Methionine**	B	1.33×10^{-4}	2.30×10^{-6}	187.66

* Molecular activity is defined as the moles of product formed per min per mole of enzyme.
** In ATP-PP_i exchange reaction.

Kinetic properties: The V and Km values for the aminoacid and tRNA as well as the molecular activities (turnover numbers) for the enzymes A and B are given in Table 2. The enzyme A had similar affinity for both $tRNA_m^{Met}$ and $tRNA_f^{Met}$ whereas enzyme B showed much lower Km value for $tRNA_f^{Met}$ than for $tRNA_m^{Met}$. The A and B forms did not differ in their Km for methionine; the enzymes also exhibited similar turnover numbers with much higher value for aminoacid than for tRNA.

DISCUSSION

The aminoacyl-tRNA synthetases, in general, range in molecular weight from 47,000 to 200,000 daltons and are monomeric or polymeric molecules [1,12]. The methionyl-tRNA synthetase of *E. coli* is a tetramer of molecular weight 180,000 daltons whereas the A and B forms of methionyl-tRNA synthetase from *M. smegmatis* reported here, are low molecular weight-proteins. The enzyme A is a dimer with subunits of 31,000 daltons and the enzyme B is a tetramer with two subunits of 31,000 daltons and two subunits of 10,000 daltons [11]. Both these forms catalyse aminoacylation as well as ATP-PP_i exchange reactions.

The two forms of methionyl-tRNA synthetase, however, show differences in their capacity to recognise the cognate tRNAs. The B enzyme aminoacylates $tRNA_f^{Met}$ to a greater extent than $tRNA_m^{Met}$ or the total tRNA, but the A enzyme shows equal activity with either species of tRNA. Therefore, the enzyme B may indeed recognise only $tRNA_f^{Met}$ whereas enzyme A can recognise both the species. Thus the addition of a protein factor (MW 20,000 daltons) to the enzyme A may modify the enzyme recognition pattern, making it specific to $tRNA_f^{Met}$, the initiator species. The distinction between the

different isoacceptor species of tRNA may become a desirable step in the regulation of translation, especially in cases like methionine and formylmethionine specific tRNAs, because of the differences in their functions. In the case of methionyl-tRNA synthetase of *E. coli* [13-16] no such multiplicity has been observed and a single enzyme could recognise both $tRNA_m^{Met}$ and $tRNA_f^{Met}$.

The possibility that the forms A and B of methionyl-tRNA synthetase from *M. smegmatis* arose due to proteolysis cannot be ruled out at this stage. However, a marker enzyme studied, viz., valyl-tRNA synthetase, purified simultaneously in this system did not show any changes in its elution profiles and hence the involvement of proteolytic cleavage appears remote.

The overall properties of the two enzymes were similar to the other well studied aminoacyl-tRNA synthetases reported in literature [1,12]. Nonetheless, the type of multiplicity due to physical presence or absence of a subunit altering the specificity towards the isospecies of tRNA, as exhibited by the methionyl-tRNA synthetase from *M. smegmatis*, reported here, is novel.

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