Initiator tRNA for the Synthesis of Globin Peptides

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A species of tRNA capable of accepting methionine and apparently structurally related to $tRNA_F^{Met}$ from E. coli is bound on ribosomes from rabbit reticulocytes incubated with NaF. As isolated the $tRNA^{met}$ is sensitive to mild oxidation by periodate and appears to be bound on the ribosomes in the deacylated form, that is without methionine. Ribosomes bearing this $tRNA^{met}$ incorporate valine into the aminoterminal position of globin peptides without detectable incorporation of aminoterminal methionine. It is concluded that the initiation of globin peptides involves a species of methionine-accepting tRNA analogous to $tRNA_F^{Met}$ in bacteria and suggested that the deacylated species of this tRNA is involved in normal initiation of globin peptides.

The role of N-formylmethionyl-tRNA in the initiation of bacterial protein synthesis has been documented by many laboratories (1). However, with the exception of mitochondria, N-formylmethionyl-tRNA has not been detected in mammalian tissues (2,3). Nascent globin peptide chains attached to ribosomes isolated from rabbit reticulocytes do not carry N-formylmethionine as the aminoterminal residue (3,4,5). Rich and his coworkers (6) and recently Arnstein and his colleagues (4) have demonstrated that rabbit globin chains can be initiated with 2-hydroxy-3-methyl butanoic acid-tRNA derived by deamination of valyl-tRNA with nitrous acid. Normal rabbit α and β globin chains have valine as their aminoterminal amino acid (7).

A possible explanation for the apparent absence of N-formylmethionyl-tRNA from mammalian tissues is provided by the hypothesis that AUG is not a universal codon for chain initiation. This proposal suggests that the initiation reactions are mediated by codons other than AUG and involve species of tRNA other than $tRNA_F^{Met}$.

Several lines of evidence appear to favor the hypothesis that the AUG codon and a species of tRNA analogous to $tRNA_F^{Met}$ are involved in chain ini-

tiation in higher organisms. When added to cell free systems derived from E. coli, RNAs from several plant virus have been shown to direct the synthesis of peptides initiated with N-formylmethionine (8). Furthermore, Caskey and his coworkers have demonstrated the presence of two species of methionyl-tRNA in guinea pig liver (9). One of these species can be formylated by E. coli transformylase. This transformylase is specific for met-$tRNA_F^{Met}$. Similarly, two species of methionine-accepting tRNA, one of which may be formylated, have been observed in tRNA from yeast (10) and wheat germ (11). Smith and his coworkers have reported involvement of a formylatable species of met-tRNA in peptide initiation on ribosomes from mouse ascites tumor cells (12). They suggest that this species of met-tRNA provides an aminoterminal methionine residue and is functionally analogous to N-formylmethionyl-tRNA from bacteria.

In an effort to identify the species of tRNA involved in the initiation of globin peptides on reticulocyte ribosomes, we have studied the species of tRNA bound to ribosomes in the presence of various inhibitors of peptide initiation in rabbit reticulocytes. Here we report a preferential accumulation of a species of tRNA capable of accepting methionine on ribosomes isolated from reticulocytes incubated in the presence of inhibiting concentrations of NaF.

NaF appears to inhibit one or more steps in peptide initiation in reticulocytes (13,14). Under the conditions of incubation with NaF and subsequent ribosome isolation used here, polysomes are converted to monomeric ribosomes of about 80S with the concomitant completion and release of globin peptide chains. Ribosomes from rabbit reticulocytes incubated with NaF were prepared as previously described (13) then washed by sedimenting them through a 5% to 20% linear sucrose gradient containing 10 mM tris·HCl, pH 7.5, 35 mM KCl and 1.0 mM $MgCl_2$. Pelleted ribosomes were resuspended then subjected to phenol extraction of soluble RNA as described earlier (3). Generally, the RNA preparations were subjected to hydrolysis at pH 9.2 to remove amino acids or pep-

tides from the tRNA. The proportion of tRNA and 5S ribosomal RNA in each preparation was determined spectrophotometrically by scanning polyacrylamide gels at 260 nm following electrophoresis (15). Aliquots of the soluble RNA preparations containing 15 μg of RNA were assayed for amino acid acceptor capacity as previously described (3). Transfer RNA was isolated by a similar procedure from ribosomal preparations containing polysomes with nascent globin chains prepared from reticulocytes incubated without NaF. The amino acid acceptor capacities of these RNA preparations and a tRNA preparation from whole reticulocytes are presented in Table I. The distribution of amino acid acceptor capacity of tRNA isolated from polysomes is similar to the amino acid composition of globin chains. Of eighteen amino acids tested, only the acceptor capacity for methionine is significantly higher on ribosomes from NaF treated cells as compared to ribosomes from untreated cells. This apparent enrichment has been five to fifteen fold in most experiments. Acceptor capacity for most amino acids is decreased in tRNA from NaF ribosomes relative to that from polysomes. On the basis of charging activity we estimate that approximately 50 percent of the tRNA recovered on ribosomes from reticulocytes incubated with NaF has the capacity to accept methionine. Other experiments not reported here indicate that after acylation of this tRNA with methionine it may then be partially formylated with an E. coli transformylase enzyme system. The lack of $tRNA^{Val}$ enrichment suggests valine as val-tRNA, to form the amino terminus of globin peptides, is not bound preferentially to these ribosomes. Thus it would appear that in the reticulocyte system NaF blocks a step in the series of initiation reactions between the binding of the initiator tRNA, "$tRNA_F^{Met}$", and the binding of the first aminoacyl-tRNA to the ribosome.

The data of Table II indicate that the methionine accepting species of tRNA obtained from ribosomes isolated from cells incubated with NaF is not acylated with either methionine or N-formylmethionine. Following incubation of the intact reticulocytes with NaF, the cells were lysed and the lysate was adjusted rapidly to pH 6.5. The ribosomes were recovered and washed

Table I

Accumulation of Methionine Accepting tRNA on NaF Ribosomes

Amino Acid	Acceptor Capacity: Reticulocyte tRNA pmoles aa/mg	% total	Regular Ribosome tRNA pmoles aa/mg	% total	NaF Ribosome tRNA pmoles aa/mg	% total
Methionine	1340	5.9	3800	6.6	15850	48.0
Alanine	3040	13.2	7150	12.4	450	4.0
Arginine	1035	4.5	1500	2.5	500	1.5
Asparagine	450	1.9	1000	1.7	950	2.9
Aspartate	130	0.6	350	0.6	425	1.3
Glutamine	320	1.4	480	0.8	350	1.1
Glutamate	700	3.1	1400	2.4	630	1.9
Glycine	2210	9.7	2600	4.5	190	0.6
Histidine	2420	10.6	6150	10.7	2360	7.2
Isoleucine	445	1.9	720	1.3	775	2.3
Leucine	2280	10.0	11340	19.7	2020	6.1
Lysine	1160	5.1	1690	2.9	1810	5.5
Phenylalanine	1370	6.0	3870	6.7	1260	3.8
Proline	160	0.7	425	0.7	315	1.0
Serine	1550	6.8	3740	6.5	790	2.4
Threonine	1560	6.8	3440	6.0	910	2.8
Tyrosine	395	1.7	1170	2.0	280	0.9
Valine	2270	10.0	6670	11.6	2220	6.7

Amino acid acceptor capacity was measured using 15 µg of the indicated tRNA preparation. The incubation mixtures contained 20 mM Tris-HCl, pH 7.5, 10 mM GSH, 2 mM ATP, 10^{-5} M of the indicated ^{14}C amino acid (100 µc/µmole) and 0.5 mg of protein from a 40-70% ammonium sulfate fraction prepared from a post-ribosomal reticulocyte lysate. Acceptor capacity was measured in 0.5 ml incubation mixtures containing optimum concentrations of $MgCl_2$ and KCl as determined for each amino acid. Incubation was carried out for 15 minutes at 37°, the mixtures were precipitated with cold 5% trichloracetic acid and then filtered. Duplicate incubations not containing added tRNA were also performed. This blank value, usually representing less than 15% of the experimental value, was subtracted. Amino acid acceptor capacity was calculated after correcting for the amount of 5S RNA present in the tRNA preparations. The reticulocyte tRNA contained 8% 5S ribosomal RNA, regular ribosome tRNA contained 62% 5S RNA and NaF ribosomal tRNA contained 68% 5S RNA. A solution containing 1 mg RNA per ml was assumed to have an absorbancy of 24 at 260 nm.

through pH 6.5 sucrose gradients. The tRNA was isolated as described above except that the pH was held below 7 at all times to minimize base catalyzed

Table II

The Effect of Periodate Oxidation on the Acceptor Capacity of Ribosomal tRNA for Methionine

	Methionine Acceptance pmoles/µg tRNA		
	Untreated	IO_4^-	IO_4^- + hydrolysis
Met-tRNA*	1420	62	870
NaF Ribosome tRNA	13,600	81	156

* Unfractionated reticulocyte tRNA charged with methionine

Methionine acceptor capacity was assayed as described in the legend to Table I. Incubation mixtures contained 6 mM $MgCl_2$ and 8 mM KCl. Untreated reticulocyte tRNA was acylated with methionine (Untreated, 1420 pmoles methionine/mg) and then periodate treated. A preparation of tRNA isolated from NaF ribosomes under slightly acidic conditions was periodate oxidized without first acylating it with methionine. In a separate experiment the acceptor capacity of this tRNA for methionine was measured as 13,600 pmoles methionine/mg (untreated). Periodate oxidation of the NaF ribosomal tRNA and reticulocyte met-tRNA was performed by the addition of 0.1 volume of 0.10 M Sodium metaperiodate to a 0.2 mg/ml solution of the tRNA in 0.10 M Potassium acetate. After incubation for 10 minutes at 0° the excess periodate was reduced by the addition of a 100 fold excess of glucose. The oxidized tRNAs were recovered by precipitation with cold 95% ethanol. A portion of each of these preparations was assayed for methionine acceptance (IO_4^-). A second portion was subjected to deacylation by incubation for 15 minutes at 37° in 0.1 M NH_4OH, pH 10.0. These preparations were then recovered by ethanol precipitation and assayed for methionine acceptance (IO_4^- + hydrolysis).

hydrolysis of any aminoacyl-tRNA present in the mixture. Portions of this tRNA were then treated with periodate under conditions known to destroy acceptor capacity of deacylated tRNA for methionine (3), and then subjected to mild alkaline hydrolysis. The amino acid of aminoacyl-tRNA protects tRNA from inactivation by periodate under the conditions used. Acceptor capacity for methionine was checked at each step as indicated in Table II. Over 98 percent of the capacity to accept methionine was destroyed by treatment of the NaF ribosomal tRNA preparation with periodate. After periodate oxidation methionine acceptor capacity was not increased by a mild alkaline deacylation procedure. Thus, the tRNA appears to have been isolated from the ribosomes

in the deacylated form. As a control, unfractionated reticulocyte tRNA was charged with methionine then added to preparations of ribosomes before phenol extraction and run in parallel experiments with the ribosomal tRNA from reticulocytes incubated with NaF. As anticipated, the acylated methionine protected a substantial portion of the $tRNA^{met}$ from periodate oxidation as shown by the regeneration of methionine acceptor capacity by deacylation of the periodate treated preparation.

As discussed above, neither methionine nor formylmethionine has been detected as an aminoterminal residue of nascent globin peptides. However, the possibility that aminoterminal methionine was present initially on these peptides then removed before the analysis could be completed was not precluded by these experiments. This possibility was tested further by measuring methionine and valine incorporation on ribosomes from reticulocytes incubated with NaF. Virtually all of the globin peptide synthesis on these ribosomes involves the incorporation of valine into the aminoterminal portion (13). Both the α and β chains of rabbit globin have valine as their aminoterminal amino acid. Methionine does not occur in native globin in the aminoterminal portion of either the α or the β chain (7). The results presented in figure 1 indicate that at least 20 times more valine than methionine is incorporated into peptides during the initial seconds of the cell-free incubation during which the aminoterminal portions of the α and β peptides are formed. A value of the valine to methionine ratio near unity during the initial portion of the time course would indicate incorporation of aminoterminal methionine.

It is conceivable, but appears unlikely, that methionine was incorporated into the aminoterminal position in the initiated chains but removed before it could be detected in these experiments. Apparently, this would have involved either charging of the deacylated "$tRNA_F^{Met}$" with methionine while it was bound to the ribosomes or exchange of the tRNA with met-tRNA during the brief incubation. An alternative possibility is that met-tRNA was bound initially to the ribosomes in the intact reticulocytes but that the methionine was subse-

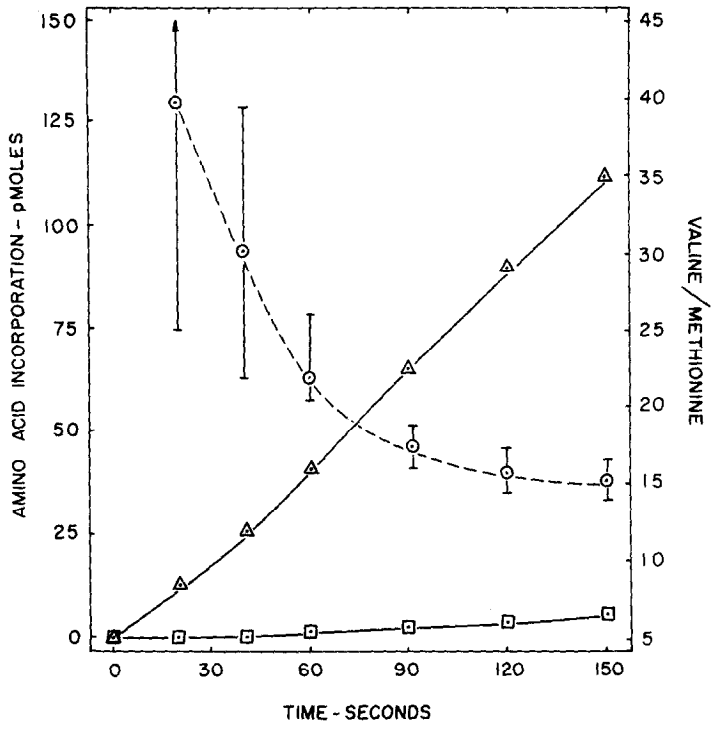

Figure 1 Time course for valine and methionine incorporation into the aminoterminus of globin peptides. Amino acid incorporation was carried out in 0.7 ml reaction mixtures containing 56 mM Tris-HCl, pH 7.5, 70 mM KCl, 3.5 mM $MgCl_2$, 30 mM GSH, 1.4 mM ATP, 0.35 mM GTP, 14 mM creatine phosphate, 50 µg creatine kinase, 200 µg deacylated rabbit liver tRNA, 1 mg 40-70% ammonium sulfate reticulocyte enzyme fraction and 2 mg of unwashed NaF ribosomes. Methionine incorporation was measured in mixtures containing 1.4×10^{-5} M ^{14}C-methionine (100 µc/µmole) and an equivalent concentration of unlabeled valine. Valine incorporation was measured in the presence of 1.4×10^{-5} M ^{14}C-valine (100 µc/µmole) and an equivalent concentration of unlabeled methionene. The assay mixtures were brought to temperature by incubating for 1 minute in the absence of ribosomes, the ribosomes were then added rapidly and incubation was carried out for the indicated time. Incorporation was stopped by the addition of 4 mg of bovine serum albumin carrier protein and 10 volumes of 5% trichloroacetic acid. The precipitated material was washed as described previously (13). The vertical bars show the range of Val/Met incorporation observed in five experiments.

—⊡——⊡— Methionine incorporation

—△——△— Valine incorporation

—⊙---⊙— Valine incorporation/methionine incorporation

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quently removed from the bound tRNA. This deacylation might have occurred in the intact cells as a consequence of inhibition by NaF. In this case, the deacylated "$tRNA_F^{Met}$"·ribosome·mRNA complex must be capable of rapid chain

initiation in order to give the results presented in figure 1.

We favor a third possibility encompassed by the hypothesis that the initial reactions of globin initiation normally involve a species of deacylated tRNA analogous to E. coli $tRNA_F^{Met}$. The reactions required for formation of the initial ribosome·mRNA·"$tRNA_F^{Met}$" complex and phasing of mRNA in the reticulocyte system may be analogous to those that occur in bacteria except that they are carried out with deacylated "$tRNA_F^{Met}$" in place of N-formyl-methionyl-tRNA. In the poly U directed reticulocyte transfer system, the initial reactions of polyphenylalanine synthesis require the codon directed binding of deacylated $tRNA^{phe}$ to the ribosome (3,16,17). We have proposed previously that in higher organisms the requirement for N-acylated aminoacyl-tRNA may be replaced by a requirement for a specific species of deacylated tRNA (3, 16). The observations presented here appear to support this proposal.

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