THE FORMATION OF HYDROXYPYRUVYL-tRNA

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1. Introduction

As part of a study on the regulation of protein synthesis during the morphogenesis of *Myxococcus xanthus*, we examined the possibility that amino acids other than methionine might be utilized as chain initiators. By analogy with *N*-formylmethionyl-tRNA [1] we reasoned that if other amino acids were involved in chain initiation, cell extracts might be able to catalyze the formation of the corresponding *N*-blocked aminoacyl-tRNA. When preliminary experiments indicated that extracts from either *M. xanthus* or *E. coli* catalyzed the conversion of serine into a derivative of ser-tRNA which lacked a free α-amino group, the problem was pursued further. The evidence presented here demonstrates that the serine derivative is the deaminated product, hydroxypyruvyl-tRNA.

2. Experimental

Transfer RNA was prepared from frozen cells of *E. coli* strain W as described previously [2]. *E. coli* MRE-600 and Q 13 enzyme extracts were prepared by centrifuging crude cell extracts for 5 hr at 105,000 × *g*, removing nucleic acids by precipitation with 0.5% streptomycin, and collecting the precipitate following addition of $(NH_4)_2SO_4$ (0.5 gm/ml). After dialysis the enzyme preparations were stored at −20° in 50% glycerol. *E. coli* MRE-600 aminoacyl-tRNA synthetase fractions, prepared in this manner, were the gifts of M.Revel and A.Danon. *M. xanthus* strain FB was grown and induced to form microcysts as described previously [3]. Crude extracts of *M. xanthus* were obtained by centrifuging sonically disrupted cells at 30,000 × *g* for 30 min. Radioactive [^{14}C] amino acids were purchased from New England Nuclear Corporation; the [^{14}C] serine had a specific activity of 123 mc/mmole. [^{14}C] *N*-formylmethionine and [^{14}C] *N*-formylserine were synthesized by the method of Sheehan and Yang [4]. *N*-acyl-aminoacyl-tRNA hydrolase [5] was prepared from *E. coli* as previously described [6].

The standard incubation mixture for charging tRNA with an amino acid contained 0.1 M Tris-HCl, pH 7.4; 0.05 M KCl, 0.015 M Mg acetate; 0.005 M dithiothreitol; 0.004 M ATP; 2 mg/ml tRNA; 3 μc/ml [^{14}C] amino acid. The reaction was initiated by the addition of enzyme fraction. After 10 min incubation at 37°, the tRNA was precipitated with trichloroacetic acid (TCA); the attached amino acid and its altered derivative were released by hydrolysis in NH_4OH and analyzed by ethyl acetate solubility [7] or electrophoresis. The procedures were as follows:

(1) 0.01 ml and 0.1 ml aliquots were placed onto Whatman 3 MM filter paper discs, precipitated with ice-cold 10% TCA, and washed with TCA, ethanol-ether (1:1), and ether. Radioactivity in discs containing the smaller aliquots was measured directly in a Packard Tricarb scintillation counter. The other discs were placed in conical centrifuge tubes containing 1 ml 0.5 M NH_4OH. After hydrolysis at 37° for 30 min, the tubes were chilled and 0.2 ml formic acid and then 1.5 ml ethyl acetate were added. The tubes were agitated on a vortex mixer and centrifuged briefly; 1.0 ml of the ethyl acetate phase was then added to 10 ml Bray's solution [8] for counting.

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(2) 0.2 ml aliquots were precipitated and washed extensively with ice-cold 10% TCA and 95% ethanol; the precipitate was then dissolved and hydrolyzed in 0.05 ml 1 M NH_4OH for 30 min at 37°. After removal of any insoluble material, the hydrolysate was applied to Whatman 3 MM paper and dried with a stream of cold air; electrophoresis was performed in 0.05 M pyridine-acetate buffer, pH 3.5, at 54 V/cm for 45 min at 4°. Radioactive materials on electrophoretograms were detected by cutting 2 cm strips and determining the radioactivity in the liquid scintillation counter.

For structural studies a mixture of ser-tRNA and hydroxypyruvyl-tRNA was purified as follows: after the standard incubation, 1.0 ml was chilled and 0.2 ml of 20% potassium acetate, pH 5, was added and the mixture was then extracted twice in the cold with 2 ml of water-saturated phenol. The aqueous phase was then precipitated with 4 ml of ethanol and washed twice with ethanol. The pellet was then dissolved in 2% potassium acetate and the tRNA was again precipitated and washed with ethanol, dissolved in 1.0 ml water, divided into 5 portions, lyophilized and stored at −20°.

3. Results

A survey experiment utilizing the standard incubation mixture and ethyl acetate extraction test revealed that of 15 amino acids examined (ala, arg, asp, glu, gly, his, ileu, leu, lys, met, phe, ser, thr, tyr and val) only methionine and serine were converted to an acid-insoluble material which was also ethyl acetate extractable after alkaline hydrolysis and acidification, indicating the absence of a free amino group. The yield of methionine product (presumably *N*-formylmethionine) was 23 ± 5% and serine derivative 2.1 ± 0.6%. Similar data were obtained using extracts from *E.coli* MRE-600, *E. coli* Q 13, *M. xanthus* vegetative cells or *M. xanthus* mycrocysts.

The serine reaction product was then examined by electrophoresis. After the reaction mixture was extracted with phenol and precipitated several times with ethanol, a portion was hydrolyzed with NH_4OH and subjected to electrophoresis (fig. 1, closed circles). The major peak, serine, migrated slightly towards the cathode, whereas a second peak, comprising approximately 4% of the input radioactivity, migrated more rapidly toward the anode than standard *N*-formylmethionine.

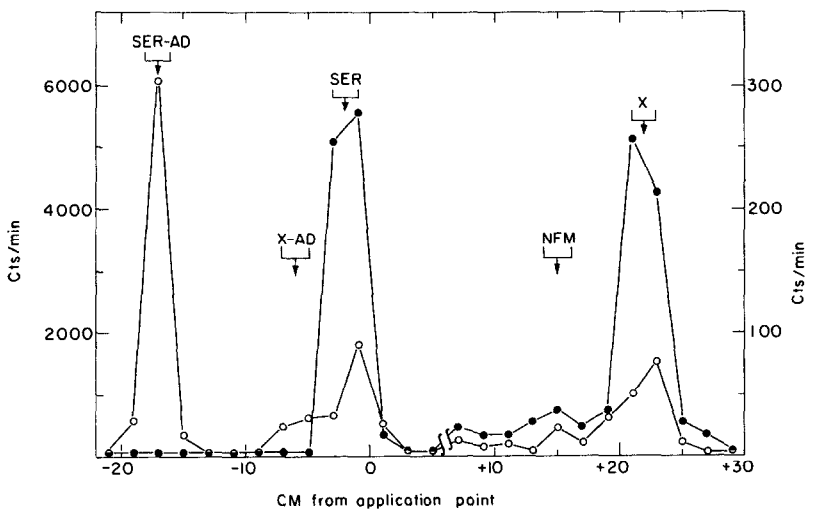

Fig. 1. Electrophoretic mobility (pH 3.5, 54 V/cm for 45 min) of (•) alkaline and (○) RNase hydrolyzed [^{14}C] seryl-tRNA containing about 4% [^{14}C] X-tRNA, prepared from 0.2 ml of standard reaction mixture as described under Experimental. Arrows indicate the mobilities of standards: serine, seryl-adenosine ester, and *N*-formylmethionine. Note that on the anode side the ordinate is expanded by a factor of 20.

This acidic component, referred to as X, was soluble in ethyl acetate at low pH. In addition, several experiments indicated a very minor peak at +15 cm, which might be *N*-formylmethionine or *N*-formylserine [9].

In order to determine whether or not compound X was joined to the tRNA, a second portion of the purified reaction mixture was treated with 10 μg/ml RNase for 30 min at 37° and analyzed by electrophoresis (fig. 1, open circles). The major peak was seryl-adenosine. Although the postulated compound X-adenosine did not separate completely from free serine, a shoulder from −3 to −7 cm was observed, which gave rise to compound X after alkaline hydrolysis. It followes that compound X must be joined to the tRNA, probably to the terminal adenosine residue. The half-life of the linkage between compound X and tRNA in 0.1 M Tris-HCl, pH 8.8, at 37° was 58 min compared to 6.3 min for ser-tRNA under identical conditions. This is consistent with the increase in alkaline stability that is commonly observed after the α-amino group is blocked or removed, e.g., phenyllactyl-tRNA versus phenylalanyl-tRNA [10].

The acidic nature of compound X further suggested that the conversion of serine into compound X involved either the loss or blockage of the α-amino group. Of several possible reaction products tested (table 1), only hydroxypyruvic acid had the same electrophoretic mobility at pH 3.5 as compound X. Radioactive compound X and authentic hydroxypyruvic acid also travelled together during electrophoresis at pH 6.5 and during paper chromatography in water-saturated phenol.

In order to confirm that compound X is hydroxypyruvic acid, a mixture of [^{14}C] ser-tRNA and [^{14}C] X-tRNA was added to a known quantity of nonradioactive hydroxypyruvic acid and hydrolyzed, and the 2,4 dinitrophenylhydrazone was prepared [11]. Of an initial 41,000 cpm, 648 cpm or 1.6% were found in the first crystals of the derivative (table 2). Since the yield of hydroxypyruvate phenylhydrazone was 36%, the fraction of input radioactivity that was hydroxypyruvic acid was 4.4%. This value is similar to that obtained by electrophoresis. Two subsequent recrystallizations, each from a different solvent, did not alter the specific activity. Thus, the unknown compound X is hydroxypyruvic acid.

The requirements for the conversion of [^{14}C] serine into [^{14}C] ser-tRNA and [^{14}C] hydroxypyruvyl-tRNA are shown in table 3. The production of both products was dependent upon Mg^{++}, ATP, enzyme fraction, and tRNA. Although the enzyme concentration was optimal for the formation of hydroxypyruvyl-tRNA, it was at least ten times more concentrated than needed for maximal production of ser-tRNA. Thus, by working with a low enzyme concentration, it was possible to obtain ser-tRNA which contained less than 0.5% hydroxypyruvyl-tRNA. The requirement for a high concentration of enzyme fraction for the formation of hydroxypyruvyl-tRNA could not be replaced by heated (5 min at 80°) enzyme. Decreasing the

Table 1
Electrophoretic mobilities at pH 3.5.

Compound	$R_N = \frac{\text{mobility of compound}}{\text{mobility of } N\text{-formylmethionine}}$
Unknown compound X	1.49
Serine	−0.13
N-formylserine	1.07
Pyruvic acid	2.52
Oxalic acid	2.92
Lactic acid	0.67
Glyceric acid	0.93
Hydroxypyruvic acid	1.49
Seryl-*O*-phosphate	1.20
Glycollic acid	0.89

Table 2
[^{14}C] hydroxypyruvate 2,4-dinitrophenylhydrazone from [^{14}C] X-tRNA.

2,4-dinitrophenylhydrazone	mg	cpm	cpm/mg
1st crystals[a] (from HCl)	15.2	648	42.6
2nd crystals, m.p. 160–162° (from ethyl acetate)	3.6[b]	160	44.4
3rd crystals (from ethyl acetate-ligroin)	1.2[b]	47	39.1

[a] Hydroxypyruvate (15 mg) was added to 1.0 ml of purified reaction mixture containing 41,000 cpm. After the mixture was hydrolyzed for 15 min in 0.1 M KOH at 30°, 44 mg of 2,4-dinitrophenylhydrazine in 2.0 ml of 2 N HCl were added. After standing in the cold overnight the crystals were collected, washed with cold 1 N HCl and water, and dried *in vacuo* over P_2O_5. Controls containing pure [^{14}C] serine in place of the reaction products or pyruvic acid in place of hydroxypyruvic acid gave no radioactivity in the corresponding 2,4-dinitrophenylhydrazones.
[b] The mass was calculated from its absorbance at 445 nm in chloroform, utilizing an extinction coefficient determined with the first crystals.

tRNA concentration resulted in a proportional decrease in both products. The fact that excess non-radioactive hydroxypyruvate did not lower the incorporation of [^{14}C] serine into [^{14}C] hydroxypyruvyl-tRNA demonstrated that the serine was modified after it was activated. Over 90% of both ser-tRNA and hydroxypyruvyl-tRNA were made acid-soluble by RNase treatment. In a separate experiment it was shown that hydroxypyruvyl-tRNA is not a substrate for *N*-acyl-aminoacyl-tRNA hydrolase. This is consistent with the reported specificity of this enzyme, which hydrolyzes *N*-substituted aminoacyl-tRNA's [5,6,12] but not the deaminated species phenyllactyl-tRNA [13].

4. Discussion

The above data indicate that serine can be enzymatically converted into hydroxypyruvyl-tRNA. The modification of serine appears to occur after its initial attachment to a specific tRNA, since free hydroxy-

Table 3
Factors influencing the formation of [^{14}C] hydroxypyruvyl-tRNA.

Modification	ser-tRNA (cpm)	hydroxypyruvyl-tRNA (cpm)
Complete[a]	26,100	608
$-Mg^{++}$, ATP, or enzyme	400	10
−tRNA	1,740	14
+RNase (10 μg/ml)	1,260	45
$\frac{1}{2}$ tRNA (1 mg/ml)	12,300	347
$\frac{1}{10}$ enzyme (0.5 mg/ml)	23,700	70
2X enzyme (10 mg/ml)	19,400	572
2X (^{14}C)serine (6 μc/ml)	28,600	619
+200 X cold serine (5×10^{-3} M)	540	15
+ Cold hydroxypyruvate (5×10^{-3} M)	24,710	698
+ 10-formyl-tetrahydrofolate	27,500	523

[a] The complete reaction mixture (2.0 ml) contained: 0.1 M Tris-HCl, pH 7.4; 0.05 M KCl; 0.015 M Mg acetate; 0.005 M dithiothreitol; 0.004 M ATP; 2 mg/ml tRNA; 3 μc/ml [^{14}C] serine; 5 mg protein/ml enzyme fraction. After incubation at 37° for 10 min, the reaction was terminated by addition of 10% TCA and analyzed by electrophoresis as described in Experimental. Modifications are described in the table. After RNase was added, the reaction mixture was incubated for an additional 10 min.

pyruvate is not an intermediate in the reaction. The crude preparations of aminoacyl-tRNA synthetases employed presumably contain an enzyme which catalyzes the reaction. However, proof for this mechanism must await the separation of the ser-tRNA synthetase from the postulated ser-tRNA transaminase or oxidative deaminase. The finding that a maximum of 5% of the ser-tRNA could be converted into hydroxypyruvyl-tRNA suggests the possibility that the transformation is limited to only one of the four serine tRNAs [14]. Again, the confirmation of this suggestion must await the separate examination of the different serine tRNAs. Until these points are clarified and hydroxypyruvyl-tRNA is demonstrated *in vivo*, it would be premature to speculate on the biological function of the substance.

It should be noted, finally, that our finding extends the list of amino acids which can be enzymatically modified while attached to tRNA, other instances being the well-documented formylation of met-tRNA [1], the reported formylation of ser-tRNA [9], and the conversion of glutamyl-tRNA to glutaminyl-tRNA [15].

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