Purification of pseudouridylate synthetase I from Salmonella typhimurium

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ABSTRACT

Pseudouridylate synthetase from Salmonella typhimurium has been purified 1000 fold and is about 90% pure. The enzyme has a molecular weight of 50,000 daltons. In the presence of tRNA there is a change in molecular weight from 50,000 to 100,000. This change does not seem to be due to the formation of a tRNA-enzyme complex but rather to a tRNA induced dimerization. Other properties of the enzyme are described.

INTRODUCTION

In prokaryotes tRNA genes are transcribed as polynucleotide precursor molecules which undergo a maturation process thereby giving rise to a mature tRNA molecule (1). This maturation process consists of size reducing nucleolytic reactions and nucleoside modifications. Significant advances in our knowledge of tRNA biosynthesis come from the isolation of mutants which have alterations in some steps of the maturation pathway and consequently accumulate maturation intermediates which, in some cases, have been purified and characterized (2-9).

HisT mutants of Salmonella typhimurium lack the pseudouridylate synthetase I, the enzyme responsible for the pseudouridine modification in the anticodon region of several tRNA (7,10). tRNA extracted from hisT mutants has been used as substrate for the in vitro synthesis of pseudouridine (10). In this paper we report the purification and initial characterization of pseudouridylate synthetase I from Salmonella typhimurium.

MATERIALS AND METHODS

Bacterial strains. TA265 is a wild-type strain of S. typhimurium LT-2, TA253 is an isogenic strain carrying the hisT 1504 mutation.

Growth of the cells and preparation of the cell extract. Cells were grown...
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in N.B. medium containing 8% nutrient broth (Difco) and 1.5% NaCl until 1 O.D. unit at 650 nm and were pelleted by centrifugation and stored frozen at -20°C. Cell extract was prepared as described (10).

Chemicals. (5-3H) uridine, (3H) tyrosine, (14C) tyrosine were purchased from the Radiochemical Centre, Amersham. Streptomycin sulphate was obtained from Squibb, DEAE cellulose from Whatman and RPC-5 absorbent was a gift from Dr. Bocchini.

Pseudouridylate synthetase assay. (5-3H) uridine labeled tRNA from strain TA253 was prepared as described (10). The tritium release assay was performed as described (10), with the difference that the incubation mixture contained 5 mM mercaptoethanol. 1 unit of pseudouridylate synthetase I is the amount of protein capable of synthesizing 1 picomole of pseudouridine in 10 min.

Reversed-phase column chromatography. Crude aminoacyl tRNA synthetases from strain TA253 were prepared according to Kelmers et al. (11) and the tRNA was aminoacylated and chromatographed as previously described (12).

RESULTS

Source of enzyme. In a preliminary investigation we have established that the specific activity of pseudouridylate synthetase I in S. typhimurium is independent of the growth medium. Cells grown in a rich medium (N.B. medium) or in minimal medium (V.B. medium, ref. 13) with glucose or citrate as a carbon source, all contained the same amount of pseudouridylate synthetase I. However the phase of growth is important because logarithmically growing cells contained two fold more enzyme than cells in the stationary phase. Pseudouridylate synthetase is a soluble enzyme, all the activity being recovered in the supernatant of an high speed centrifugation which removed ribosomes from the cell extract. We therefore used, as a starting material for our purification, cells grown in N.B. medium and harvested in the late log phase. Cells were disrupted by sonication and the high speed supernatant was prepared as described (10). The specific activity at this stage was 20-30 units/mg of proteins.

Purification of pseudouridylate synthetase I. Pseudouridylate synthetase I was precipitated by addition of 20 mg/ml of streptomycin sulphate. Recovery of the enzyme activity was about 70% with a specific activity of 200 units/mg of proteins. It should be emphasized that the optimal
concentration of streptomycin sulphate used for precipitating the enzyme changes significantly from preparation to preparation and has to be determined each time by a trial experiment. The streptomycin sulphate precipitate was resuspended in Buffer A (10 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol) containing 0.33M NaCl, at a protein concentration of 50 mg/ml, followed by the addition of 450 mg/ml of ammonium sulphate. After centrifugation the precipitate was resuspended in Buffer A and desalted by G-25 sephadex chromatography. This gives a 60% recovery of the enzymatic activity with a specific activity of 400-600 units/mg of proteins. The ammonium sulphate fraction was applied to a DEAE cellulose column (100 mg of proteins/cm³ of resin). The flow rate was half of the bed volume per hour. After washing with two column volumes of Buffer A a linear gradient of NaCl (from 0 to 0.185M) was applied. The enzymatic activity eluted at about 50 mM NaCl. The fractions containing the enzyme were pooled and ammonium sulphate was added to reach 70% saturation. The specific activity of this preparation was about 4000 units/mg of proteins. The precipitate was resuspended in Buffer A and applied to a G-100 Sephadex column. The elution profile for which is shown in Fig. 1. The main peak of activity elutes at about 50,000 M.W., but a minor peak is always present in a region of 10,000 - 15,000 M.W. The relative amounts of these two peaks of activity varied significantly from preparation to preparation. We rechromatographed on a second G-100 column the two activities separately and could see no reequilibration between the two forms, each one eluting in the second chromatography at the same position as in the first. This shows that they are not readily interconvertible (data not shown). The specific activity of the first peak was about 25,000 units/mg of proteins, with a purification factor of about 1000 fold and an overall yield of 10-15%. The enzyme is not yet homogeneously pure because in a 12.5% SDS polyacrylamide gel electrophoresis we observe a main band of about 55,000 M.W., which we assume is pseudouridylate synthetase, and several minor bands constituting about 10-15% of the total proteins loaded (Fig. 2). The enzyme from peak 1 was precipitated with ammonium sulphate (70% saturation) and stored at 4°C at a concentration of 10 mg/ml in Buffer A. histT 1504 tRNA\textsuperscript{Tyr} is converted in vitro to wild-type tRNA\textsuperscript{Tyr} by pseudouridylate synthetase I. tRNA molecules, extracted from histT 1504 mutant and therefore lacking pseudouridine in the anti-codon region, elute behind the corresponding wild-type pseudouridylated species on
Figure 1. Sephadex G-100 chromatography of pseudouridylate synthetase I.
The enzyme was applied onto the column (2.3 x 90 cm) at a concentration of
1 mg/ml. The column was run at a flow rate of 7 ml/hour and 1.5 ml
fractions were collected. Enzymatic activity was assayed on 10 µl
aliquots.

Factors which influence the reaction. The in vitro synthesis of pseudo-
uridine occurs without the addition of any cofactor (10,14). In our
search for compounds which might influence the rate or the extent of the
in vitro pseudouridine synthesis we have found that none of the following
compounds in a concentration range between 10^{-6} M to 10^{-3} M has any
influence on the reaction: ribose, ribose-1-phosphate, ribose-5-phosphate,
ribose-5-phosphate, uracil, uridine, uridylic acid, folic acid, vit. B12,
pyridoxal phosphate, pyridoxine, biotin, thiamine. Reducing agents,
such as mercaptoethanol, do not have any effect on the activity of freshly
prepared pseudouridylate synthetase I, but are very effective in restoring
Figure 2. 12% polyacrylamide gel electrophoresis. 10 µg of proteins were applied on a slot of a 20 cm x 20 cm polyacrylamide slab gel prepared according to Laemmli (13) and run at 60 V for 5 hours.

the original activity of aged enzyme preparations, as shown in Fig. 4. Possibly SH groups play an essential role for the activity of the enzyme as it is also suggested by the sensitivity of the enzyme to iodoacetamide: in about 5 minutes all the activity is lost (Fig. 5).

Wild-type tRNA was found to be an inhibitor of the reaction. Fig. 6 shows the percent of residual activity in the presence of increasing concentration of unlabeled wild-type tRNA: at 5 µM there is a 50% inhibition.
Figure 3. RPC-5 column chromatography.
Cochromatography on reversed-phase column N.5 (RPC-5) of histT tRNA charged with \(^{3}H\) tyrosine and in vitro modified histT tRNA with \(^{14}C\) tyrosine was performed as described (9). Wild-type tRNA, charged with \(^{3}H\) tyrosine was chromatographed in a separate experiment. • histT tRNA, O in vitro modified histT tRNA, ■ wild-type tRNA.

Figure 4. Activation with mercaptoethanol.
Enzymatic activity was measured as described in Materials and Methods. 0 enzyme activity after storage for 40 days at \(4^\circ\)C, • same as before after preincubation with 15mM mercaptoethanol, A enzyme activity of freshly prepared pseudouridylate synthetase I.
Figure 5. Inhibition of enzymatic activity by iodoacetamide. The enzyme was incubated at 37°C in the presence of 150 mM iodoacetamide. 1 ul aliquots were used for enzymatic assay. The time on the abscissa refers to the period of incubation in the presence of iodoacetamide.

Isoelectric focusing. In attempts to further purify pseudouridylate synthetase we observed that the enzyme can be subjected to isoelectric focusing and also retains its activity in the presence of ampholines. Although the isoelectric focusing is redundant for purification purposes, it provides information about the isoelectric point of the enzyme. Fig. 7 shows that, after the DEAE cellulose step, the enzyme has an isoelectric point at pH 4.1.

Glycerol gradient sedimentation. G-100 Sephadex chromatography indicated that pseudouridylate synthetase activity can be found in two forms, distinguished by their size. The larger and more abundant has an M.W. of 50,000 and the other of less than 15,000. We have studied the molecular weight of the enzyme by glycerol gradient sedimentation. Fig. 8 shows the activity profile of pseudouridylate synthetase 1 on a 10-30% glycerol gradient, at various stages of the purification. Panel a shows that in the high speed supernatant the enzyme activity is recovered in a peak corresponding to about 100,000 daltons. The enzyme has approximately the same molecular weight after streptomycin sulphate precipitation (Fig. 8,
Figure 6. Enzyme inhibition in the presence of wild-type tRNA. Enzymatic activity was measured as radioactivity released after 4 minutes of incubation in the presence of increasing amounts of wild-type tRNA.

pabel b) and after ammonium sulphate precipitation (Fig. 8, panel c). A drastic change, however, is observed following DEAE cellulose chromatography, the activity being recovered in a peak of about 55,000 molecular weight. Apparently the enzyme exists as a 100,000 M.W. complex which is dissociated after DEAE cellulose.

Since tRNA absorbs strongly to DEAE cellulose, it seemed possible that the 100,000 to 55,000 M.W. transition was due to the removal of tRNA bound to the enzyme. In fact preincubation of the 55,000 M.W. enzyme in the presence of tRNA converted the enzyme back to 100,000 M.W., as it is shown in Fig. 9, panel a. In the experiment shown, wild-type S. typhimurium crude tRNA was used but the results are identical with hist tRNA or with several different purified tRNA species from Salmonella or even from calf thymus. On the other hand preincubation with poly U (average length of about 100 nucleotides) does not affect the M.W. of the enzyme.

The increase in the molecular weight of the enzyme could be due to
Figure 7.  Electrofocusing of pseudouridylate synthetase I.
The enzyme, 800 µl at a protein concentration of 14 mg/ml, was added to
the light solution and the electrofocusing was performed as described (22)
for 14 hours.  Initial conditions were 420 V and 16 mA, final conditions
were 500 V and 2 mA.  Ampholine pH range was from 3.5 to 10.  Fractions
were neutralized and the enzyme activity measured on 5 µl aliquots.

------Absorbancy at 280 nm, O enzyme activity, pH.

the formation of a tRNA-enzyme complex or, alternatively, to a tRNA
induced complex formation, for instance a dimerization of the enzyme.
This last hypothesis appears more probable in the light of the results
obtained when (5-3H) uridine labeled tRNA was used.  Fig. 9, panel b
shows the sedimentation of the pseudouridylate synthetase I, preincubated
with (3H-uridine) labeled tRNA.  The enzyme activity (continuous line) is
found in the 100,000 M.W. region of the gradient, whereas all the radio-
activity (dotted line) sedimented in the 4S region, in coincidence with
the sedimentation profile of tRNA run in a parallel gradient (panel c).

DISCUSSION

Pseudouridylate synthetase I from S. typhimurium has been purified
about 1000 fold, and is about 90% pure as judged by gel electrophoresis.
The purified enzyme is able to convert hist tRNA into wild-type tRNA
(Fig. 3) and can be used for pseudouridylation of in vitro transcribed
tRNA^Tyr precursor molecule (14).  The enzyme can apparently exist in more
than one form, as judged by molecular weight, the more abundant form
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Figure 8. Glycerol gradient sedimentation.
Glycerol gradients were from 10% to 30% in buffer A. 12 ml gradients
were run in Beckman SW41Ti rotor for 48 hours at 4°C.
O enzymatic activity.

having a molecular weight of 55,000 daltons. It is an acidic protein,
like many other proteins interacting with nucleic acids, with an iso-
electric point at pH 4.1.

An interesting property of the enzyme is its apparent change in
molecular weight upon interaction with tRNA. Our results suggest a tRNA
induced change in the quaternary structure may be a dimerisation of the
55,000 M.W. subunit into a 100,000 M.W. dimeric complex. We do not know
whether this molecular weight change plays any role in the course of
pseudouridine synthesis. Since both pseudouridylated and unpseudo-
uridylated tRNA induce the molecular weight change, the possibility that
the reaction was reversible was considered. That this is not the case,
least under our experimental conditions, is shown by the results
presented in Fig. 3: wild-type tRNA remains unmodified after incubation
with pseudouridylate synthetase. Wild-type tRNA is, however, an inhibitor
of the biosynthesis of pseudouridine in vitro. The relevance of this
phenomenon in vivo remains to be clarified.

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The reaction catalyzed by pseudouridylate synthetase I occurs by a mechanism which is still obscure. We know that during the reaction there is no exchange of uracil, uridine or uridylic acid (G. Ciliberto, unpublished), and therefore it is unlikely that the excision of uracil is an intermediate step in the conversion of uridine into pseudouridine. The reaction occurs without the addition of any cofactor, however, as the reaction in vitro is only 25% complete it is possible that the conditions which we use are not optimal. We have used a crude hist tRNA preparation as a substrate for the reaction but it is likely that the natural substrate is a precursor molecule. In fact pseudouridine has been found in the sequence of dimeric tRNA precursor (5). Moreover, the in vitro transcribed tRNA tyr precursor is a substrate for pseudouridylate synthetase I (14).

About 50% of all tRNA species in E. coli (and in S. typhimurium which seems to be identical in this respect) have pseudouridine in the anticodon region (15). An inspection of the various sequences in the
Tablo 1. Nucleotide sequence surrounding pseudouridine in the anticodon region of E.Coli, Salmonella typhymurium and phage T(f tRNA. N stands for unspecified nucleotide.

\[ \text{Anticodon region of tRNA} \]

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The anticodon region (Table I) suggests that the pseudouridylate synthetase I does not recognize a specific sequence but rather is capable to convert into pseudouridine any uridine residue which happens to be present in the primary sequence of the anticodon region, at position B, C, or D, as indicated in Table I. It is noteworthy that there are other constraints in the sequence of this region: for instance Adenosine is never found in position B and Guanosine is never found in position D.

We do not know the reason for these regularities in the nucleotide sequence of the 3' side of the anticodon region. With respect to the conversion of uridine into pseudouridine, we know that it always occurs (tRNA^Val^2 is probably a special case) but it is not essential for the function of tRNA as adaptor of aminoacids. HisT mutant tRNA's which lack pseudouridine in this region functions perfectly well in the aminocacylation reaction and in protein synthesis (17,18). On the other hand these mutant tRNA are unable to participate in the transcriptional regulation of several operons (10,12), suggesting that pseudouridine is important for the role of tRNA in regulation (19).

This cannot however be a general rule. For instance, tRNA^Trp^ is also involved in the regulation of transcription but (20,21), not having an uridine residue in the primary structure (15), does not, of course, have a pseudouridine in the anticodon region. We think that pseudouridine per se is not essential for the regulatory function but rather the presence of a uridine residue in the anticodon region is incompatible with
the regulatory role, hence the necessity to convert it to pseudouridine.

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*The one exception to this rule is tRNA^Val^2 which has an uridine residue in position D. However this tRNA is exceptional also because the A residue flanking the 3' side of the anticodon is not modified. Maybe the anticodon region of tRNA^Val^2 has some unusual features.

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REFERENCES

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