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Notes & Tips

A thin-layer electrophoretic assay for Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase

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The availability of a complete and accurate set of aminoacylated transfer RNAs (tRNAs)[1] is fundamental to protein biosynthesis in all three kingdoms of life [1]. Despite this rigid requirement, many bacteria and archaea lack asparaginyl-tRNA synthetase (AsnRS) and/or glutaminyl-tRNA synthetase (GlnRS), the canonical enzymes that catalyze the direct biosynthesis of Asn-tRNA^{Asn} and Gln-tRNA^{Gln} [1]. In organisms lacking either or both of these enzymes, the requisite aminoacyl-tRNAs are biosynthesized indirectly via a two-step process (Fig. 1). First, a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) or a nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) generates Asp-tRNA^{Asn} or Glu-tRNA^{Gln}, respectively. Second, the bifunctional Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (Asp/Glu-Adt or GatCAB) catalyzes a transamidation reaction to repair both of these misacylated tRNAs. A Glu-tRNA^{Gln}-specific amidotransferase (Glu-Adt or GatDE) is also used in some species instead of, or in addition to, Asp/Glu-Adt. A recently published review described these amidotransferases [2]. Asp/Glu-Adt typically is assayed by thin-layer chromatography (TLC) to separate the different amino acid products [3]. This assay is time-consuming and cannot distinguish between Asp and Gln or between Asn and Glu. Here we report an improved assay for Asp/Glu-Adt that uses thin-layer electrophoresis (TLE) [4,5], instead of TLC, for amino acid separation. The electrophoretic step is rapid and leads to improved resolution.

Although the overall reaction catalyzed by Asp/Glu-Adt is the simple conversion of an amino acid carboxylate (in either Asp or Glu) into a carboxyamide (in Asn or Gln), this transformation takes place while the amino acid is attached to a tRNA, making product detection complicated because of the large size of the aminoacyl-tRNA substrate compared with the small chemical change introduced by the enzyme. A typical assay for Asp/Glu-Adt begins with either ^{14}C-labeled Asp-tRNA^{Asn} or ^{14}C-labeled Glu-tRNA^{Gln} [6]; these radiolabeled aminoacyl-tRNAs are generated using the corresponding ND-AspRS or ND-GluRS. Each of these tRNAs is incubated with Asp/Glu-Adt, ATP, and nonradiolabeled glutamine as the ammonia donor. Time points are removed and quenched in 3 M sodium acetate (pH 5), followed by phenol extraction. Each quenched aliquot, containing a mixture of unreacted ^{14}C-labeled Asp-tRNA^{Asn} or ^{14}C-labeled Glu-tRNA^{Gln} and of enzyme-generated ^{14}C-labeled Asn-tRNA^{Asn} or ^{14}C-labeled Gln-tRNA^{Gln}, is deacylated by mild alkaline treatment, and the released amino acids are subsequently separated by TLC and visualized by phosphorimaging of the ^{14}C-labeled amino acids. Asp/Glu-Adt has also been assayed by HPLC after amino acid derivatization with a fluorescent label to facilitate detection; this method is not as sensitive as the standard TLC/radioactivity method described above, but it has the advantage that it does not require radioactivity [7].

Because of the polar nature of free amino acids, the TLC plates are developed in highly aqueous solvents (solvent A: isopropanol:formic acid:acetic acid:water in an 8:1:1:4 ratio [Fig. 2A]) and take 1–2 h for complete elution. As can be seen in the TLC plate shown in Fig. 2A, these conditions effectively resolve Asp ($R_f = 0.63$) from Asn ($R_f = 0.70$) and resolve Glu ($R_f = 0.70$) from Gln ($R_f = 0.60$). The drawbacks to this method are the time it takes to develop the TLC plate; the fact that Asn and Glu (Fig. 2A, lanes 2 and 4) and Asp and Gln (lanes 1 and 5) have identical and

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[1] *Abbreviations used:* tRNA, transfer RNA; AsnRS, asparaginyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; ND-AspRS, nondiscriminating aspartyl-tRNA synthetase; ND-GluRS, nondiscriminating glutamyl-tRNA synthetase; Asp/Glu-Adt or GatCAB, Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase; Glu-Adt or GatDE, Glu-tRNA^{Gln}-specific amidotransferase; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis.

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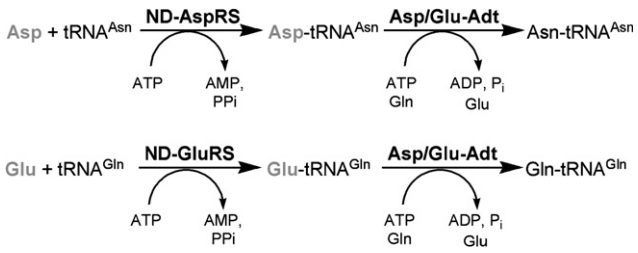

Fig. 1. Biosynthesis of Asn-tRNA^{Asn} and Gln-tRNA^{Gln} in the absence of AsnRS and GlnRS. Top panel: An ND-AspRS catalyzes the biosynthesis of Asp-tRNA^{Asn}. Next, Asp/Glu-Adt converts Asp-tRNA^{Asn} into Asn-tRNA^{Asn} in an ATP-dependent reaction that uses glutamine as a nitrogen donor. Bottom panel: Gln-tRNA^{Gln} is biosynthesized in the same two-step process, beginning with an ND-GluRS (or GluRS2 [11,12], not shown). In both cases, misacylation is shown in gray.

nearly identical retention factors, respectively; and occasional streaking. Because Asp/Glu-Adt is confronted with both Asp-tRNA^{Asn} and Glu-tRNA^{Gln} in vivo in many organisms [8–10], an assay that allows the resolution of all four relevant amino acids would be highly useful for experiments designed to mimic in vivo conditions.

We decided to evaluate TLE as a technique for better amino acid resolution in Asp/Glu-Adt assays. In TLE, compounds are eluted electrophoretically, and consequently the charged states of the different amino acids contribute to their mobility across the stationary phase. We first evaluated TLE separation of Asp, Asn, Glu, and Gln in the absence of Asp/Glu-Adt (Fig. 2B) using standard TLC plates (precoated silica gel 60_{F254} plate, Fisher Scientific). Square plates were cut to dimensions suitable for elution in a standard submerged horizontal gel electrophoresis chamber (e.g., a 15-cm wide × 7-cm long TLC plate in a Bio-Rad Mini-Sub Gel GT). Each amino acid was loaded separately along a line at the center of the plate (the origin, Fig. 2B), and the plate was gently loaded with running buffer (solvent B: 0.1 M NaH_2PO_4, pH 7). The plate was placed in the electroelution chamber, and the anode and cathode chambers were filled with running buffer. Filter paper was soaked in running buffer and used to bridge the buffer beds at each electrode with the TLC plate. The plate was subjected to 100 V for 40 min and then was developed by exposure to an enhanced PhosphorImager screen (Amersham Biosciences), and imaged on a Molecular Dynamics Storm 840 PhosphorImager. As can be seen in Fig. 2B, TLE led to clear resolution of Asp ($R_f = +0.20$) and Asn ($R_f = -0.34$) and of Glu ($R_f = +0.14$) and Gln ($R_f = -0.43$), with better separation and less streaking than were obtained by standard TLC. TLE also led to the resolution of Asn and Gln (cf. lanes 2 and 5 in Fig. 2B and R_f values given above) and the partial resolution of Asp and Glu (cf. lanes 1 and 4 and R_f values given above). Furthermore, TLC analyses are hindered by occasional diffusion and streaking of the amino acid; these problems were not observed in any of our TLE experiments. Therefore, TLE is superior to TLC for its ability to resolve all four amino acids.

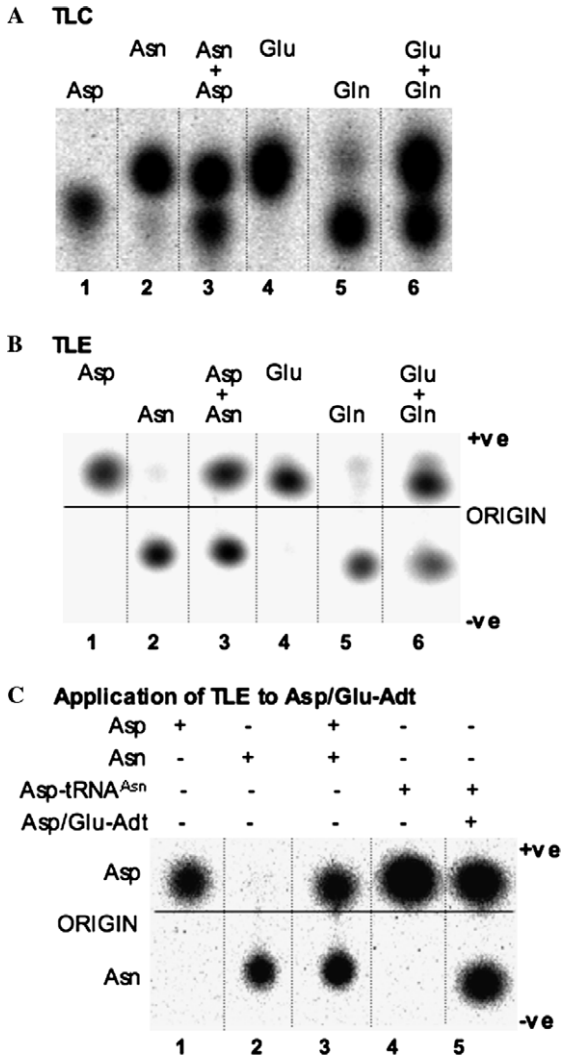

Fig. 2. TLE as a method to assay Asp/Glu-Adt. (A) Phosphorimage of a TLC plate developed in solvent A for 2 hours. The origin is not shown and is approximately 2.25 in. below the lowest spot. (B) TLE plate electroeluted for 40 min at 100 V in solvent B. The solid line denotes the site of sample loading (origin); the directions of the cathode (+) and the anode (−) are also labeled. In both panels A and B, each lane was spotted at the origin with 50 nCi of the relevant ^{14}C-labeled amino acid, as labeled: lane 1, Asp; lane 2, Asn; lane 3, 1:1 mixture of Asp/Asn; lane 4, Glu; lane 5, Gln; lane 6, 1:1 ratio of Glu–Gln. (C) Assay of Asp/Glu-Adt using TLE. Asp/Glu-Adt was assayed as described in the text. Lanes 1–3 represent amino acid controls (lane 1: Asp; lane 2: Asn; lane 3: 1:1 ratio of Asp–Asn). Lane 4 shows the results from the no-enzyme control where the Asp-tRNA^{Asn} remains unreacted. Lane 5 shows the results from the Asp/Glu-Adt assay where approximately 50% of the Asp-tRNA^{Asn} has been converted to Asn-tRNA^{Asn}.

Next, we verified TLE as a method to assay Asp/Glu-Adt (Fig. 2C). *Helicobacter pylori* Asp/Glu-Adt (310 nM) was incubated with ^{14}C-labeled Asp-tRNA^{Asn} (2.5 μmol, 25 μM, 500 μCi), 4 mM ATP, 1 mM Gln, 4 mM $MgCl_2$, and 1.25 mM KCl in 10 mM Hepes (pH 7.5) for 10 min. A parallel no-enzyme control experiment was also conducted. The reactions were quenched and precipitated. The resultant pellets were resuspended in 50 μl of 25 mM KOH and deacylated at 65 °C for 10–15 min. Following deacylation, mixtures were neutralized with 12.5 μl of 0.1 M HCl and

pellets were dried in a Savant SC110A Speed Vac Plus. Pellets were resuspended in 7 μl H_2O and analyzed by TLE (3-μl spots) as described above. As can be clearly seen in Fig. 2C (cf. lanes 4 and 5), Asp/Glu-Adt converted approximately 50% of the Asp-tRNA^{Asn} into Asn-tRNA^{Asn} and the radiolabeled amino acids from this reaction were resolved successfully by TLE.

In summary, we have reported the development of a TLE assay for Asp/Glu-Adt. This assay is facile, reproducible, and faster than the standard TLC assay typically used with this enzyme.

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