A broadly applicable continuous spectrophotometric assay for measuring aminoacyl-tRNA synthetase activity

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ABSTRACT

We describe a convenient, simple and novel continuous spectrophotometric method for the determination of aminoacyl-tRNA synthetase activity. The assay relies upon the measurement of inorganic pyrophosphate generated in the first step of the aminoacylation of a tRNA. Pyrophosphate release is coupled to inorganic pyrophosphatase, to generate phosphate, which in turn is used as the substrate of purine nucleoside phosphorylase to catalyze the *N*-glycosidic cleavage of 2-amino 6-mercapto 7-methylpurine ribonucleoside. Of the reaction products, ribose 1-phosphate and 2-amino 6-mercapto 7-methylpurine, the latter has a high absorbance at 360 nm relative to the nucleoside and hence provides a spectrophotometric signal that can be continuously followed. The non-destructive nature of the spectrophotometric assay allowed the re-use of the tRNAs in question in successive experiments. The usefulness of this method was demonstrated for glutamyl-tRNA synthetase (GlnRS) and tryptophanyl-tRNA synthetase. Initial velocities measured using this assay correlate closely with those assayed by quantitation of [³H]Gln-tRNA or [¹⁴C]Trp-tRNA formation respectively. In both cases amino acid transfer from the aminoacyl adenylate to the tRNA represents the rate determining step. In addition, aminoacyl adenylate formation by aspartyl-tRNA synthetase was followed and provided a more sensitive means of active site titration than existing techniques. Finally, this novel method was used to provide direct evidence for the cooperativity of tRNA and ATP binding to GlnRS.

INTRODUCTION

The formation of aminoacyl-tRNA by aminoacyl-tRNA synthetases (AARS) is described as a two-step reaction (1,2). Firstly, the amino acid (AA) is activated by forming enzyme-bound aminoacyl-AMP with the release of pyrophosphate (PP_i)

Secondly, the activated amino acid esterifies the terminal ribose of the tRNA to yield AA-tRNA and AMP

In the case of glutamyl-, glutamyl- and arginyl-tRNA synthetases formation of the aminoacyl adenylate (equation 1) requires the presence of the cognate tRNA.

There are two widely used assays (3,4) for these reactions. The first is the usual aminoacylation assay, which measures AA-tRNA as trichloroacetic acid (TCA)-precipitable radioactivity from radiolabeled AA in the presence of tRNA and ATP. The second assay (ATP-PP_i exchange) measures a combination of the forward and backward reactions of equation 1 by measuring the formation of radioactive ATP from [³²P]PP_i in the presence of the synthetase, ATP and AA. These assays suffer from a number of disadvantages, namely both are discontinuous and, consequently, not particularly sensitive to deviations from linearity in kinetic measurements. While these problems can be overcome using stopped flow kinetics, this technique requires considerably more enzyme than steady-state kinetics.

We have devised a new assay which addresses some of these issues. Our assay continuously monitors PP_i production, the first step catalyzed by aminoacyl-tRNA synthetases in the forward direction. Essentially, AARS-dependent PP_i production is monitored in the presence of excess inorganic pyrophosphatase to generate 2 mol phosphate (P_i) per mol aminoacyl-AMP formed. Phosphate production is then coupled to the phosphorylation of the chromogenic nucleoside 2-amino 6-mercapto 7-methylpurine ribonucleoside (AMMPR) by excess purine nucleoside phosphorylase to yield ribose 1-phosphate and 2-amino 6-mercapto 7-methylpurine (AMMP). The AMMP base has a high absorbance at 360 nm relative to the corresponding nucleoside, AMMPR (5), and so provides a spectrophotometric signal that can be continuously followed. The reaction scheme is summarized below.

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We have applied this system to assay the activities of *Escherichia coli* glutamyl-, tryptophanyl- and aspartyl-tRNA synthetases (GlnRS, TrpRS and AspRS).

MATERIALS AND METHODS

Reagents

[³H]Glutamine (45 Ci/mmol) and [¹⁴C]tryptophan (53.8 mCi/mmol) were obtained from Amersham. AMMPR was synthesized and purified as described (5). The UV and NMR spectra of our preparation agreed with previously reported data for this compound (5,6). Unfractionated *E. coli* tRNA was depleted of inorganic phosphate by repeated ethanol precipitations of aqueous solutions. *Escherichia coli* tRNA^{Gln} and tRNA^{Glu} were overexpressed and purified as described (7,8). Unmodified *E. coli* tRNA^{Trp} was prepared as the run-off transcript and further purified as described by Rogers *et al.* (9). Yeast inorganic pyrophosphatase (IPP), calf spleen purine nucleoside phosphorylase (cs-PNP) and bacterial purine nucleoside phosphorylase (b-PNP) were obtained from Sigma. b-PNP was further purified 11-fold by chromatography on Mono Q Sepharose. *Escherichia coli* GlnRS and TrpRS were purified as described (10,11). AspRS was a gift of Dino Moras. One enzyme unit is defined as the amount of enzyme required to generate 1 μmol/min AA-tRNA at 23°C.

Spectrophotometric determinations

Spectrophotometric assays for GlnRS, TrpRS and AspRS activity were conducted at 23°C in 2 mm width, 10 mm path length quartz cuvettes in a Perkin-Elmer Lambda-2 dual beam UV/visible light spectrophotometer. The assay (0.1 ml) contained 30 mM HEPES-KOH, pH 7.6, 10 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 5 mM Gln (or 1 mM Trp) and tRNA^{Gln} or tRNA^{Trp} at concentrations specified in the text, PNP (final concentration 10 U/ml), IPP (final concentration 10 U/ml) and 0.15 mM AMMPR. Upon addition of the final component (usually synthetase, tRNA or the AA) absorbance changes (A₃₆₀) were measured against a reference cuvette lacking this component.

To recover and regenerate the tRNA used in the spectrophotometric assay the assay mix was extracted with phenol (saturated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the aqueous phase was extracted with chloroform:isoamyl alcohol (24:1 v/v) and the nucleic acid was precipitated with ethanol. The precipitated tRNA was deacylated in 20 mM Tris-HCl, pH 9.0, at 37°C for 20 min, precipitated again and dissolved in water.

Aminoacylation with radiolabeled amino acids

Standard *in vitro* aminoacylation assays were performed at 23°C in a total volume of 0.1 ml. All components listed in the spectrophotometric assay mix were added (see above). For GlnRS activity measurements tRNA and AA concentrations were 5.03 μM and 0.75 mM [³H]Gln (sp. act. 13 400 c.p.m./nmol) respectively. The reaction was initiated by addition of enzyme; controls were performed in the absence of either tRNA or enzyme. Every 2 min 20 μl aliquots were removed and spotted onto Whatman 3MM disks, which were then processed for liquid scintillation counting of [³H]aminoacyl-tRNA as described (12). TrpRS activity assays contained 1 mM [¹⁴C]Trp (sp. act. 6350 c.p.m./nmol) and 0.8 mg/ml tRNA^{Trp} transcript. To reduce non-specific binding of the labeled free amino acid, the filters

used for precipitation of Trp-tRNA were soaked in 10 mM unlabeled Trp and dried before use and the trichloroacetic acid solution contained 5 mM Trp (13).

Active site titration

Active site titration was performed as previously described (14), except that the appropriate AA was substituted for tyrosine as detailed below.

5,5-Dithiobis-2-nitrobenzoic acid (DTNB) treatment of GlnRS

Modification of thiol groups of GlnRS was performed by incubating 2.6 μM protein with 0.5 mM DTNB at 23°C in 20 mM Tris-HCl, 10 mM MgCl₂, pH 8.0. At various time intervals aliquots were diluted 10-fold with 20 mM Tris-HCl, 10 mM MgCl₂, pH 8.0, and assayed immediately for activity using the spectrophotometric assay. Rates obtained were linear, suggesting that dilution had quenched the reaction of DTNB sufficiently for it to be negligible over the time course of an assay. Incubations were also carried out supplemented with ATP, tRNA^{Gln} or tRNA^{Glu}, as described in the text.

RESULTS

Response of AMMPR to pyrophosphate and phosphate

Determination of the magnitude of the extinction coefficient of the PNP-dependent cleavage of AMMPR (A₃₆₀ increase) in the presence of PP_i and IPP or inorganic phosphate alone was necessary to prove the correct response of the assay system, as suggested in equations 4 and 5. Incubations were carried out in assay buffer, 0.2 mM AMMPR, 0.2 U/ml PNP and either increasing concentrations of 0.2 U/ml IPP together with increasing concentrations of PP_i or phosphate alone. The final changes in A₃₆₀ were then recorded. The response of the assay system to both PP_i and phosphate concentration proved to be linear (Fig. 1). As expected, the A₃₆₀ at a given PP_i concentration was 1.95-fold that at the same phosphate concentration. Therefore, the extinction coefficients calculated for the A₃₆₀ on PP_i or phosphate additions were 19 500/M/cm and 10 000/M/cm respectively. The latter value is in excellent agreement with that of Webb (5). In addition, as the stoichiometry of the IPP reaction is correct, i.e. a given PP_i concentration generates twice the A₃₆₀ increase as the same phosphate concentration, the system described in equations 4 and 5 works accurately to quantitate PP_i.

Spectrophotometric detection of GlnRS activity

Addition of GlnRS to a cuvette containing all the assay components induced an absorbance increase (A₃₆₀). The initial absorbance change was linear with time (Fig. 2A). To ascertain that the increase in A₃₆₀ was in fact GlnRS specific, the dependence on the presence of Gln and tRNA^{Gln} was also determined. No increase in A₃₆₀ could be detected if Gln was substituted by Glu or if tRNA^{Gln} was substituted by tRNA^{Glu} (data not shown).

IPP was essential for PNP catalyzed AMMPR cleavage in the GlnRS assay (equation 5); if cs-PNP was used instead of b-PNP, no increase in absorbance was seen due to contaminating IPP activity in the bacterial enzyme. However, if after 1 min IPP was added to the cuvette, a jump in A₃₆₀ corresponding to the PP_i

Figure 1. Dependence of increase in A_{360} on phosphate or PP_i in the presence of IPP due to cleavage of AMMPR. Incubation with either phosphate or PP_i in the presence of IPP was performed as duplicates as described in Materials and Methods. A_{360} dependence on [phosphate] (●) or [PP_i] (○).

accumulated in the absence of IPP was observed. The magnitude of the jump suggested that PP_i had been produced at the same rate before addition of IPP as after addition of IPP. These data indicated that it was indeed the PP_i released by GlnRS (equation 1) that was being measured and that IPP did not itself stimulate the rate of production of PP_i .

Further proof that the assay was monitoring GlnRS exclusively was the effect of an anti-GlnRS antibody on the measured rate. Addition of increasing concentrations of antibody progressively slowed the absorbance increases measured relative to those obtained in the absence of the antibody (H.-U. Thomann, unpublished data). Together these results demonstrate that the spectrophotometric quantitation method specifically follows the progress of a GlnRS catalyzed reaction.

Pyrophosphate release is stoichiometric with tRNA glutaminylation

It was necessary for two reasons to compare the rates measured by the spectrophotometric assay with rates obtained by measuring [3H]Gln-tRNA^{Gln} formation in determining TCA-insoluble radioactivity. According to equation 3 above, the production of PP_i should be equimolar with the generation of Gln-tRNA and thus comparison of the rates of generation of these two products should, if identical, provide the final proof that the spectrophotometric assay was exclusively detecting GlnRS activity. In addition, we wanted to provide the basis for comparison of data obtained with the spectrophotometric assay with previous data concerning this enzyme obtained by already established methods.

GlnRS activity when determined by the spectrophotometric assay was linear with added GlnRS (Fig. 3A). When Gln-tRNA was determined in the same reaction mixtures (including AMMPR, IPP and PNP) by the TCA precipitation method the rates obtained were essentially identical to those obtained by the spectrophotometric technique (Fig. 3A). Control experiments indicated that the presence of AMMPR, IPP and PNP enhanced GlnRS activity slightly (by 10%). Thus these results show that the rate of tRNA acylation is equivalent to the rate of PP_i release. Furthermore, they suggest that the coupled two-enzyme reaction

Figure 2. Spectrophotometric assay of tRNA-dependent PP_i production by AARS. (A) Chart recorder trace of GlnRS catalyzed PP_i accumulation in the presence of $46 \mu M$ tRNA^{Gln}. After a stable baseline was reached, the reaction was started with the addition of 1 nmol enzyme. (B) Time course of TrpRS catalyzed PP_i accumulation in the presence of $32.8 \mu M$ tRNA^{Trp} and 10 nM TrpRS. The reaction was started upon addition of 1 mM Trp. Data were collected at 12 s time intervals and plotted against time.

has no significant impact on the rate of tRNA charging, as the rate determining step is in fact not represented by Gln-AMP formation. Thus the use of the spectrophotometric assay and comparison with the results of the TCA precipitation method are justified.

tRNA acceptor activity as measured by the spectrophotometric assay

Total charging of a tRNA preparation with one particular AARS can be used to determine the amount of a particular acceptor tRNA. The co-linearity of enzyme activity determined by the two methods suggested that complete aminoacylation of tRNA

Figure 3. Dependence of AARS activity on protein as measured by the spectrophotometric and radioactive assays. (A and B) GlnRS and TrpRS activity dependence on protein respectively. tRNA concentrations were 5.03 and 32.8 μ M, respectively. Data collected during the spectrophotometric assay (○) are compared with those obtained by the radioactive assay (●).

species in different samples could now be assayed spectrophotometrically. The absorbance changes on addition of increasing amounts (10–80 μ g) of partially purified (30.2% glutamylation as determined by radioactive assay) tRNA^{Gln} were related to pmol PP_i generated using the extinction coefficient derived for PP_i generation (see above). The data were plotted against the amount of tRNA added. The same axis depicts the [³H]Gln-tRNA synthesized (pmol) measured by TCA precipitation. Again, the correlation between the two sets of data is very striking (Fig. 4), leading to the conclusion that the spectrophotometric assay provides a non-destructive method for the assay of extent of tRNA aminoacylation.

Figure 4. Stoichiometric concordance of tRNA charging with PP_i release. Radioactive and spectrophotometric assays were carried out as described in the text over a range of tRNA concentrations and initiated by the addition of gln to avoid any inaccuracies caused by any residual phosphate in the tRNA preparation. ○, data obtained by radioactive assay; ●, data obtained by spectrophotometric measurement.

Not only could the spectrophotometric assay be employed to measure charging of partially purified tRNA^{Gln} samples, but it could also be used to assay the acceptor activity of crude tRNA preparations from *E.coli*. Indeed, from the change in absorbance on addition of 0.35 mg of such a crude preparation, it could be calculated that the natural abundance of the two tRNA^{Gln} isoacceptors was 1.8% of the total tRNA.

tRNA recovery from the spectrophotometric assay

The spectrophotometric nature of the assay placed a lower limit on the amount of tRNA^{Gln} that could be employed in the assay. Indeed, it can be shown that with an extinction coefficient of 19 500/M/cm a final A₃₆₀ of 0.005 would require generation of PP_i equivalent to the charging of 0.25 μ M tRNA under the current assay conditions. As a result, the spectrophotometric assay requires the use of relatively large quantities of tRNA compared with the radioactive method. Therefore, in some cases this may prohibit the accurate determination of the K_m for tRNA of the synthetase. However, this potential drawback is somewhat offset by the ability to recover the tRNA used in the assay. Therefore, the several spectrophotometric assay mixtures were extracted with phenol and the tRNA purified. No tRNA degradation was detected by analysis on denaturing polyacrylamide gels. The glutamine acceptor activity of the recovered tRNA (measured by [³H]Gln-tRNA formation) was as high as the value measured spectrophotometrically in the assay from which the tRNA was reclaimed.

Application of the spectrophotometric assay to a synthetase employing tRNA-independent aminoacyl adenylate formation

To demonstrate a more general function of the spectrophotometric assay we applied this method to *E.coli* TrpRS. The addition of tRNA^{Trp} to a cuvette containing the remaining assay components

Figure 5. Tryptophanylation of tRNA by TrpRS. This was carried out as described in the text. The initially missing substrates Trp and tRNA^{Trp} were added subsequently as indicated by numbered arrows. 1, addition of Trp to 1 mM; 2 and 3, addition of 3.28 nmol each of tRNA^{Trp} transcript.

and TrpRS induced a linear absorbance increase (A_{360}). This rate also proved to be dependent on Trp addition and was directly proportional to enzyme concentration (Fig. 3B). As in the case of GlnRS (Fig. 4), the presence of AMMPR, IPP and PNP had no impact on TrpRS activity as compared with the rate of [¹⁴C]Trp-tRNA formation determined by the TCA method (data not shown).

As demonstrated for the GlnRS system, the spectrophotometric assay allowed measurement of the extent of aminoacylation of tRNA samples. The spectrophotometric results were in excellent agreement with those obtained by the TCA precipitation method. These results prove again that the spectrophotometric assay is a non-destructive method to determine tRNA acceptor activity. Interestingly, although pure tRNA^{Trp} transcript was used, the amount of PP_i determined indicated that only 10% of the tRNA^{Trp} transcript was aminoacylated. Addition of fresh tRNA to the cuvette led to the same extent of aminoacylation at the same rate, indicating that incomplete aminoacylation was not due to product inhibition (Fig. 5). This percentage of charging was confirmed by measuring [¹⁴C]Trp-tRNA formation.

However, the Trp acceptor activity was doubled if, prior to the assay, the tRNA was denatured by heating to 80°C for 2 min and renatured by slowly cooling to room temperature in the presence of 10 mM MgCl₂. This enhancement of acceptor activity by renaturation of tRNA^{Trp} has been previously observed (15,16) and indicates again that the characteristics of this tRNA as determined by the TCA precipitation method are accurately reflected in the results obtained with the spectrophotometric assay.

Active site titration of AspRS

It has previously been demonstrated that the number of catalytically competent sites within an AARS can be determined by

Figure 6. Formation of aspartyl adenylate from aspartate (2.86 mM), ATP (2 mM for the spectrophotometric assay, 5.14 μ M for the radioactive assay) and aspartyl-tRNA synthetase in the presence of pyrophosphatase. (a) A typical absorbance trace at 360 nm after mixing the reagents for the spectrophotometric assay in a stopped flow apparatus (Applied Photophysics model SX17MV) and (b) data from the conventional radioactive assay.

active site titration (14). This method measures the magnitude of the burst of aminoacyl adenylate formation by observation of ATP depletion. Here we show, using *E.coli* AspRS as a representative AARS, that the amount of PP_i produced correlates with ATP consumption during this initial burst (Fig. 6). The concentration of active sites determined by the two methods deviated by <10%. These experiments were also repeated at 50% of the enzyme concentration described here and gave appropriately lower values (data not shown).

tRNA and ATP protect GlnRS against sulfhydryl group modification

Introduction of the fluorescent probes pyrenylmaleimide and acrylodan onto the native GlnRS has been used to attempt to show cooperativity of binding between tRNA and ATP (17,18). However, as the GlnRS characteristics were investigated after the enzyme was modified with a reporter group, it is difficult to extrapolate to behavior of the native protein. Therefore, we examined the effects of various substrates on the preservation of

Figure 7. Effect of ATP and tRNA on the thiol-directed inactivation of GlnRS by DTNB. GlnRS (2.6 μ M) was either incubated alone (●) or with 0.1 mM DTNB supplemented with buffer (○), 2 mM ATP and 2 μ M tRNA^{Gln} (○), 2 mM ATP (□), 2 μ M tRNA^{Gln} (■) or 2 μ M tRNA^{Glu} (▲), respectively.

activity of the native enzyme during its inactivation by the cysteine-directed reagent DTNB, using protection of activity as an index of the binding of ATP or tRNA^{Gln} to native GlnRS.

As the spectrophotometric assay indicated, although GlnRS activity was stable, the enzyme was inactivated by DTNB with a half-life of 3 min (Fig. 7). The presence of 2 μ M tRNA^{Gln} and 2 mM ATP only marginally affected this rate of inactivation, as shown by 2.5- and 3.7-fold increases in the half-life of the enzyme (Fig. 7). However, in the presence of both substrates the rate of inactivation was dramatically attenuated, by 11-fold, to yield a half-life of 32.5 min (Fig. 7). The data are consistent with a cooperative effect between the binding of ATP and tRNA^{Gln}. To substantiate this conclusion, we repeated the latter incubation with ATP and 2 μ M tRNA^{Glu} in place of tRNA^{Gln}. Unlike tRNA^{Gln}, the non-cognate tRNA^{Glu} was incapable of potentiating the protective effect of ATP, the half-life of the enzyme being 4 min, close to that displayed by GlnRS incubated in DTNB alone.

DISCUSSION

Here we describe a sensitive continuous spectrophotometric technique to assay both AARS activity and tRNA charging as a function of PP_i generation. The PP_i released from ATP upon AARS catalyzed AA-AMP formation (equation 1) is coupled to a two-enzyme detection system. Firstly, 2 mol inorganic phosphate are formed by IPP catalyzed hydrolysis of 1 mol PP_i (equation 4). In the second coupled step the P_i is used for PNP-dependent cleavage of the chromogenic nucleoside AMMP, generating the chromophore base AMMP (equation 5). The formation of AMMP can be directly followed by the increase in absorption at 360 nm. As ATP and tRNA exhibit absorption maxima at much lower wavelengths (~260 nm), these substrates do not interfere with this assay. However, substitution of other nucleosides in this role that have previously been investigated as PNP substrates would not be desirable, as the corresponding free bases show absorption maxima in the range 260–293 nm (19).

The assay has been applied to measure the activity of two enzymes, GlnRS and TrpRS, and should be applicable to other

AARS. It is linear with respect to time and protein. As was representatively demonstrated for GlnRS, the assay displays the correct substrate dependencies and the IPP rate dependence proved that the assay followed PP_i production. It is of great importance that, at least in the cases of GlnRS and TrpRS, the constant removal of released PP_i by IPP does not affect the net charging of tRNA. These findings do not support the suggestions of Kern *et al.* (17) that the rate determining step for the GlnRS catalyzed reaction may lie with neither the transfer of the glutamyl moiety between AMP and tRNA (equation 2) nor the release of the Gln-tRNA from GlnRS, but with either PP_i release or Gln-AMP formation itself. In this context it might be interesting to examine the relationship between measurements made with the spectrophotometric assay as applied to arginyl-tRNA synthetase with corresponding aminoacylation data, as the rate limiting step for this enzyme has been unambiguously shown to be release of the charged tRNA (20).

The spectrophotometric assay of PP_i release was also employed to quantitate AA-AMP formation by AspRS. This allowed active site titration of the enzyme and the results were comparable with those obtained by the conventional radioactive assay. In the case of GlnRS the correlation between the tRNA glutamylation and PP_i release quantitation methods indicates that production of Gln-AMP and subsequent transfer of the AA onto the tRNA are tightly coupled. Interestingly, Kern *et al.* (17) showed that at the pH employed here PP_i exchange, i.e. measurement of the backward reaction (equation 1), was six times faster than aminoacylation. Similar observations have been made for other AARSs (21). In this context it is of great significance that spectrophotometric PP_i quantitation strictly follows tRNA aminoacylation rates, while the [³²P]PP_i exchange assay measures turnover of the aminoacyl adenylate (equations 1 and 2).

For GlnRS and TrpRS this is the first actual demonstration of a 1:1 stoichiometry between aminoacylation and PP_i production. This is significant because the two enzymes differ fundamentally in their mechanism of action, in that TrpRS, unlike GlnRS, does not need the cognate tRNA to catalyze aminoacyl adenylate formation (4). tRNA^{Trp} is known to be sensitive to denaturation and to be difficult to charge. Again, the correlation between the two assays was evidenced by increasing the tRNA acceptor activity by renaturation of the tRNA prior to assay (15,16).

The spectrophotometric assay was an excellent tool in a series of experiments designed to follow the response of GlnRS activity to cysteine modification with DTNB. The continuous nature of the assay made it possible to follow loss of activity during the time course of the modification itself and therefore gave information that would have been unavailable at that time by any radioactive procedure. The use of this assay also considerably simplified the performance of this experiment with regard to sampling.

That ATP and tRNA^{Gln} acted to potentiate protection of GlnRS against DTNB inactivation exerted by the other and that this effect was tRNA^{Gln} specific suggested, at least, that binding of either ligand to the native protein is necessary for binding of the other. These data extend the conclusions drawn by Battacharyya and Roy (18) and suggest a cooperativity of binding of tRNA^{Gln} and ATP to the enzyme. It will be interesting to see if this is also a feature of ArgRS and GluRS, which, like GlnRS, are novel in their requirement for PP_i exchange.

Although the spectrophotometric assay needs a 10-fold higher tRNA concentration compared with radioactive methods in order to measure a detectable absorption increase, this disadvantage is

offset by the fact that tRNA could be re-used in subsequent assays after recovery and deacylation. The assay is limited by the pH dependence of the absorbance of AMMP (5), however, this limitation can be overcome by re-calibration of the response of the assay system to PP_i at the desired pH. We discovered that *E. coli* S100 extracts degrade the free base AMMP, thus masking the A₃₆₀ increase upon PNP-dependent AMMP cleavage. However, while this clearly prohibits the use of this assay to quantify synthetase activity in cell extracts, at least for GlnRS, this activity can be separated from the peak of GlnRS activity by fractionation of a crude extract on Mono Q Sepharose (H.-U. Thomann and A. Lloyd, unpublished observation). Therefore, the spectrophotometric assay can be used to test GlnRS activity from this step onwards.

Two different continuous methods have previously been devised to monitor AARS activity. One method measures the PP_i exchange reaction catalyzed by alanyl-tRNA synthetase (22). Here the ATP analog adenylyl α -imidotriphosphate is used as the substrate to generate ATP in the presence of PP_i. ATP synthesis is then linked to hexokinase (which cannot use the imidotriphosphate) and glucose 6-phosphate dehydrogenase, which, with glucose and NAD⁺, generates NADH and thus an increase in A₃₄₀. This method, although ingenious, has the disadvantage that adenylyl α -imidotriphosphate is not a substrate, but an inhibitor, of many AARSs (23). More recently, an assay linking AMP generation through myokinase, pyruvate kinase and lactate dehydrogenase to NADH consumption has been developed for AlaRS (24). This assay, which monitors the rate described by equation (2), is more widely applicable, but suffers from the fact that it has to follow small changes against an already high initial absorbance. In contrast, the spectrophotometric assay we report here measures an increase in absorbance and therefore has a lower signal to noise ratio. Also, comparison of the extinction coefficients of NADH and of AMMP on reaction with IPP-cleaved PP_i indicates that the system described here is 50% more sensitive.

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