Rapid and selective enzymatic assay for L-methionine based on a pyrophosphate detection system

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Abstract

An enzymatic assay for L-methionine was developed by coupling adenosylmethionine synthetase (AdoMetS) to a pyrophosphate (PPi) detection system, which was constructed using pyruvate phosphate dikinase (PPDK). To expand the use of this assay, the PPI detection system was embodied as three different forms, which allowed PPi to be measured by UV, visible, and fluorescent detectors. The assay system was robust and could tolerate the addition of inorganic phosphate and ATP to the assay mixtures. L-methionine could be accurately determined by coupling the PPI detection system and AdoMetS. This AdoMetS coupling assay was highly selective to L-methionine and exhibited no significant activity to other proteinaceous amino acids, ammonia, or urea, unlike conventional enzymatic assays for L-methionine. Spike and recovery tests showed that the AdoMetS assay could accurately and reproducibly determine increases in L-methionine in human plasma samples without any pretreatment to remove proteins and potentially interfering low-molecular-weight molecules. The high selectivity and robustness of the AdoMetS assay provide rapid and high-throughput analysis of L-methionine in various kinds of analytes.

Key words

L-methionine; pyrophosphate; adenosylmethionine synthetase; pyruvate phosphate dikinase

Introduction

The quantification of amino acids is an important technique for a wide range of fields including scientific research, manufacturing industries, and medical applications. This technique has recently been attracting increasing attention because the concentrations of each amino acid in the human body can be a useful biomarker to diagnose various diseases [1; 2]. From this viewpoint, it is beneficial to develop assay methods that can easily and rapidly quantify amino acids in crude samples including biological samples. High selectivity and robustness are required for this purpose because crude samples typically contain numerous kinds of small molecules besides the target amino acid. Many instrumental methods, such as those using HPLC and LC-MS, have been reported for measuring amino acid concentrations. However, these often require pretreatments to remove proteins or other molecules prior to the analysis, and also require expensive and bulky instruments. These restrictions prevent the wider application of amino acid analysis. An enzymatic assay is one of the promising solutions [1] because it can simultaneously analyze multiple samples without any specialized, bulky, and expensive instruments.

L-Methionine is a key metabolite in carbon, nitrogen, and sulfur metabolism. This amino acid is also known to be a biomarker for homocystinuria, and thus the measurement of L-methionine in human plasma and other biological samples is one of the beneficial techniques from a medical viewpoint. Several enzymatic assays for L-methionine using methionine-γ-lyase (EC 4.4.1.11) or methionine dehydrogenase have been reported [3; 4]. In the assay with the former enzyme, ammonia produced from methionine is reacted with a fluorescent dye and determined. Although this assay has been used in mass screening for homocystinuria in Japan, it has the drawback of the contamination of ammonia. Biological samples and detection instruments are often contaminated by ammonia. Estimations using this assay become less reliable, especially when blood samples are analyzed, because the ammonia level in human blood varies from 30 to 70 µM in response to nutrient and health conditions [5]. The selectivity of this assay is also not high because methionine-γ-lyase reacts with thiol group compounds, such as L-cysteine and L-homocysteine, as well as L-methionine. The assay using methionine dehydrogenase, in which the methionine-dependent reduction of NAD is detected, overcomes these problems. However, this assay requires a pretreatment to remove branched-chained amino acids from the analytes because this enzyme shows broad reactivity toward these amino acids [4].

In order to develop a methionine-selective assay,
adienosylmethionine synthetase (AdoMetS; EC 2.5.1.6; Eq. 1), which converts L-methionine and ATP to S-adenosyl-L-methionine (AdoMet), inorganic phosphate (Pi), and pyrophosphate (PPI) [6], represented one of the promising enzymes. High substrate specificity toward methionine was expected because AdoMetS reacts with methionine at its thioether group, which is absent in the side chains of other amino acids. The activity toward methionine was expected because AdoMetS phosphorylates L-methionine and [2-3H]ATP [7; 8; 9] or by determining the AdoMet produced [10]. Although the radioisotopic methods are highly sensitive and accurate, these methods are unsuitable for rapid and simple determination of methionine. AdoMetS activity may also be measured by detecting the PPI produced, which is known to be detectable by the following two methods. In the first method, PPI is converted into ATP by ATP sulfurylase (EC 2.7.7.4) or another enzyme, and the produced ATP is subsequently determined [11]. Although this method has been commercialized as the PPIlight™ Inorganic Pyrophosphate Assay (Lonza, Basel, Switzerland), this cannot be coupled with AdoMetS because of severe interference with luciferase by the addition of ATP, which must be added to the assay mixture as a substrate of AdoMetS. The second method is pyrophosphatase (EC 3.6.1.1) treatment, which converts PPI into Pi, followed by the detection of the produced Pi [12], and this has been commercialized as the EnzChek® Pyrophosphate Assay Kit (Invitrogen, CA, USA). Although this method can be coupled with AdoMetS, the types of analytes are limited to Pi-free solution because the method cannot distinguish PPI from Pi. Since many crude samples contain high concentrations of Pi, another PPI assay tolerant to Pi was needed in order to analyze a wide range of crude samples.

In this study, we constructed a PPI assay system that could quantify PPI even in the presence of ATP and Pi, and coupled this system to AdoMetS. The coupled assay allowed the rapid and selective quantification of L-methionine.

L-methionine + ATP → S-adenosyl-L-methionine + Pi + PPI (Eq. 1)

Materials and methods

Bacterial strains

Escherichia coli JM109 and E. coli BL21 (DE3) were used as the hosts for cloning and overexpression, respectively. Propionibacterium freudenreichii NBRC 12426 and E. coli W3110 were used as the source of the pyruvate phosphate dikinase (PPDK; EC 2.7.9.1) gene (AJ549196) and AdoMetS gene (metK in AP009048), respectively.

Plasmid construction

The PPDK gene was amplified by PCR from the P. freudenreichii genome as a template using the following two primers: 5'-tatactacagtcgtcgcagctc-3' and 5'-tatactacgactactgcatcagctct-3'. The amplified fragment was inserted into pET-28a after NdeI and XhoI digestion to construct an expression plasmid. The nucleotide sequence of the inserted fragment was confirmed and modified using the QuikChange site-directed mutagenesis kit (Agilent, CA, USA) according to the AJ549196 sequence. *metK* was amplified by PCR from the *E. coli* W3110 genome as a template using the following two primers: 5'-aaactcgcagctggctggttttacagtc-3' and 5'-aagacttctcagcttcgagctc-3'. The amplified fragment was inserted into pET-28a after *NdeI* and EcoRI digestion to construct an expression plasmid.

Overexpression and purification of AdoMetS and PPDK

*E. coli* BL21 (DE3), harboring the expression plasmid for AdoMetS or PPDK, was cultured aerobically in LB medium containing 50 µg/ml kanamycin at 37°C. Gene expression was induced by adding IPTG at a final concentration of 0.5 mM when the optical density at 600 nm reached 0.6-0.8. The culture temperature during the induction shifted from 37°C to 30°C for PPDK expression. Cells were harvested by centrifugation approximately 4 hours after the addition of IPTG.

Enzyme solutions were kept at 4°C throughout AdoMetS and PPDK purification. Cells were disrupted by sonication and centrifuged to remove cell debris. The supernatant was applied to an open column packed with 5 ml of Ni-Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK). After eluting unbound proteins with 20 mM Tris-HCl, 300 mM NaCl, and 50 mM imidazole-HCl (pH 8.0), the His-tagged protein was eluted with 20 mM Tris-HCl, 300 mM NaCl, and 500 mM imidazole-HCl (pH 8.0). Glycerol was added to the purified fractions of AdoMetS and PPDK at a final concentration of 20% (v/v), and the enzymes were stored at -80°C until use.

PPDK assay for PPI detection

PPDK activity was measured by the following three methods quantifying the produced pyruvate. (i) In the ultraviolet (UV) assay, PPDK was coupled with lactate dehydrogenase (LDH; Oriental Yeast, Tokyo, Japan) to monitor NADH oxidation. Reaction mixtures contained 20 mM imidazole-HCl (pH 7.0), 10 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM potassium phosphoenolpyruvate (PEP), 0.5 mM AMP, 0.25 mM NADH, 0.5 U/ml LDH, 0-200 µM PPI, and PPDK. The mixtures were incubated at 30°C, and the decrease in absorbance at 340 nm was monitored either by a spectrometer with 1-cm path-length cuvettes or by a microplate reader (TECAN, Seestrasse, Switzerland). (ii) In the colorimetric assay, PPDK was coupled with pyruvate oxidase (POX; Toyobo, Osaka, Japan) and horseradish peroxidase (HRP; Wako, Osaka, Japan) to monitor 4-aminoantipyrine oxidation. The reaction mixture contained 20 mM imidazole-HCl (pH 7.0), 10 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM PEP, 0.5 mM AMP, 0.5 mM Na₂HPO₄, 1 mM 4-aminoantipyrine, 1 mM N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine
AdoMetS assay for L-methionine detection

The mixtures were incubated at 30°C, and the increase in absorbance at 555 nm was monitored using a microplate reader. (iii) In the fluorescent assay, PPDK was coupled with POX and HRP to monitor the production of resorufin from 10-acetyl-3,7-dihydroxyphenoxazine (ADHP; also known as Amplex Red). The reaction mixture contained 20 mM imidazole-HCl (pH 7.0), 10 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM PEP, 0.5 mM AMP, 0.5 mM Na₂HPO₄, 1 mM TOOS, 1.5 U/ml POX, 7.5 U/ml HRP, 0-100 µM L-methionine, PPDK, and AdoMetS. The mixtures were incubated at 30°C, and the fluorescence increase was monitored using a microplate reader at excitation and emission wavelengths of 530 nm and 590 nm, respectively. Absorbance or fluorescence measured at the end of the reaction was used to construct PPI standard curves. The detection limit of PPI-free blank samples (n = 20).

Robustness of the PPDK assay was investigated by constructing standard curves in the presence of Pi, ATP, or human plasma in reaction mixtures. Recovery rates were estimated from the slope of the standard curves, taking that of the standard curve constructed in the absence of these additives as 100%.

AdoMetS assay for L-methionine detection

These three types of detection methods were adopted for AdoMetS assays as well as PPDK assays. (i) UV assay mixture consisted of 20 mM imidazole-HCl (pH 7.0), 20 mM KCl, 10 mM NH₄Cl, 5 mM MgCl₂, 1 mM ATP, 0.5 mM PEP, 0.1 mM AMP, 0.5 mM Na₂HPO₄, 50 µM ADPH, 1.5 U/ml POX, 7.5 U/ml HRP, 0-10 µM PPI, and PPDK. The mixtures were incubated at 30°C, and the absorbance was measured at 555 nm using a microplate reader. (ii) Colorimetric assay mixtures contained 20 mM imidazole-HCl (pH 7.0), 20 mM KCl, 10 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM AMP, 0.5 mM Na₂HPO₄, 50 mM imidazole-HCl (pH 7.0), 20 mM KCl, 10 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM AMP, 0.5 mM Na₂HPO₄, 50 µM ADPH, 1.5 U/ml POX, 7.5 U/ml HRP, 0-100 µM L-methionine, PPDK, and AdoMetS. The selectivity of the assay was estimated in the colorimetric assay system. L-methionine in the standard assay mixture was replaced by 0.5 mM L-alanine, L-cysteine, L-aspartic acid, L-glutamic acid, L-phenylalanine, glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tryptophan, L-tyrosine, or 5 mM D-methionine.

Spike and recovery tests with human plasma

Human plasma from a single donor was purchased from Kohjin Bio (Saitama, Japan). The frozen plasma was thawed and centrifuged to remove precipitates just before use. The reaction mixtures contained 50 mM imidazole-HCl (pH 7.0), 20 mM KCl, 10 mM NH₄Cl, 5 mM MgCl₂, 1 mM ATP, 0.5 mM PEP, 0.1 mM AMP, 0.5 mM Na₂HPO₄, 1 mM 4-aminoantipyrine, 1 mM TOOS, 20% (v/v) human plasma, 1.5 U/ml POX, 7.5 U/ml HRP, PPDK, and AdoMetS. L-Methionine was added to each mixture at a final concentration of 0, 5, 10, and 20 µM (n = 6 or 12). These final concentrations corresponded to 0, 25, 50, and 100 µM increases in L-methionine in the plasma. Standard curves were constructed by assay mixtures containing no plasma, and these were used to calculate the recovery rate of the plasma-containing samples.

The intrinsic L-methionine concentration in the plasma sample was estimated from the AdoMetS-dependent increase in the absorbance. Reaction mixtures contained the above constituents and plasma, except the extrinsic L-methionine. The AdoMetS-dependent increase in the absorbance was calculated by subtracting the absorbance of negative controls devoid of AdoMetS.

Instrumental analysis of methionine in human plasma

Human plasma ultrafiltered by Amicon Ultra (Millipore, Bedford, MA, USA) was derivatized by AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and analyzed by a Waters Acquity ultra-performance liquid chromatography system as previously described [14]. The elution of derivatized methionine was detected by a fluorometer at excitation and emission wavelengths of 266 nm and 473 nm.
Designing the coupling assay for methionine determination

PPDK is an enzyme that catalyzes the reversible reaction shown in Equation 2 using PPi as one of the substrates or products. This enzyme allowed us to design three coupling assay systems for detecting L-methionine (Fig. 1). These assay systems share the coupling of AdoMetS and PPDK, in which L-methionine is consumed as a substrate producing an equimolar amount of pyruvate. To detect the pyruvate produced, different kinds of detective methods were employed in each assay system, namely, UV, colorimetric, and fluorescent detection methods. This selectability of the detection method was expected to expand the use of this assay in various analytic environments.

\[
\text{PPi + phosphoenolpyruvate + AMP} \rightleftharpoons \text{pyruvate + ATP + Pi} \quad \text{(Eq. 2)}
\]

PPDK purification and PPi detection

PPDK has been found and characterized in some bacterial, archaeal, and eukaryotic species [15; 16; 17]. A PPDK gene was cloned from \( \text{P. freudenreichii} \), a bacterium possessing one of the well-studied PPDKs [15; 18]. Recombinant PPDK was heterologously expressed in \( \text{E. coli} \) and purified by Ni-Sepharose chromatography. The specific activity of purified PPDK was estimated to be 2.3 U/mg, which is comparable to that of the reported native enzyme, 2.1 U/mg [15]. Purified PPDK was used for the construction of enzymatic assays to detect PPi as below.

The UV assay was constructed by coupling PPDK with LDH, which enabled the detection of NADH decreases by monitoring absorbance at 340 nm. After mixing all the constituents of the coupling reaction mixture, PPi-dependent absorbance decreases were observed at 340 nm. The amount of this decrease was strongly correlated to the amount of PPi added to the mixtures (Fig. 2A). This linear standard curve demonstrated that PPi can be accurately quantified by this assay. Measurements in 1-cm path-length cuvettes (not shown) estimated that PPi resulted in an absorbance decrease of 6.2 cm\(^{-1}\) mM\(^{-1}\), which indicated that almost all PPi in the mixture was effectively consumed and resulted in the oxidation of equimolar NADH. This estimation was also supported by the fact that comparable absorbance changes were observed when PPi was replaced by pyruvate at the same concentrations (not shown).

A colorimetric assay was developed as the second detection system by coupling PPDK with POX and HRP, which produce a chromogen from 4-aminoantipyrine and TOOS [13] depending on pyruvate. In this assay, the produced chromogen was detected by monitoring absorbance at 555 nm. The addition of PPi resulted in a proportional color development giving a linear standard curve (Fig. 2B). This result demonstrates that an accurate concentration of PPi can be determined by this colorimetric assay as well as the UV assay. The colorimetric PPi assay was more sensitive than the UV assay.

As the third, a fluorescent assay was developed using the same enzymes as the colorimetric assay, but the reagent used for color development was replaced by a fluorescent dye, ADHP. PPi-dependent increases in fluorescence were observed, giving a linear standard curve (Fig. 2C). It is noteworthy that sensitivity was higher in the fluorescent assay than in the UV and colorimetric assays. This enabled the highly sensitive detection of analytes containing a low concentration of L-methionine. High sensitivity is also useful for estimating low Km values. In practice, the Km of PPDK for PPi was estimated to be 44 ± 3 µM by the fluorescent assay. This low Km indicates the suitability of PPDK for PPi measurements over a broad range of concentrations. The detection limits of UV, colorimetric, and fluorescent assays were estimated to be 7, 0.3, and 0.2 µM, respectively (Table 1). Each assay reached the endpoint 5-10 minutes after the reaction started, indicating the rapidity of these assays.

Robustness of the PPi assay

Since Pi and ATP are products of the PPDK forward reaction, it was conceivable that the accuracy of PPi determination may be impaired by these molecules. To evaluate the effects of these molecules, PPi standard curves were constructed when 0-2 mM Pi and ATP were added to the assay mixtures of the three assay systems. The addition of Pi and ATP had no
significant effect on the reaction rate or on the end-point values of each assay system. The obtained standard curves (not shown) were almost the same as those constructed in the absence of Pi and ATP (Fig. 2). Recovery rates were estimated to be approximately 100% for each assay from the slopes of the standard curves (Fig. 3). This result indicates the robustness of the PPi assays, which allowed the coupling of the PPi assays to AdoMetS in the presence of Pi and ATP.

To verify whether the PPi assays were appropriate for measurements in biological samples, PPi standard curves were constructed in the presence of human plasma, an example of a biological sample. Although PPDK activity was slightly inhibited by plasma, no significant effect was observed in the UV and colorimetric assays with approximately 100% recovery (Table 1). This result demonstrates that these two assays can be applied to biological samples. The fluorescent assay showed a linear standard curve in the presence of plasma as well as the two assays. However, its fluorescence intensity was weaker than that in the absence of plasma (76% recovery) because of the attenuation of resorufin fluorescence by plasma.

**AdoMetS and PPDK coupling assay for methionine detection**

A gene encoding AdoMetS, *metK*, was cloned from *E. coli*, overexpressed, and purified. When purified AdoMetS was coupled to the PPi detection system, an L-methionine-dependent production of PPi was observed. No pyrophosphatase activity, which hydrolyzes PPi to Pi, was detected as previously reported [7], which indicates the suitability of AdoMetS for accurate and reproducible methionine measurements. The coupling assay estimated the Km value of AdoMetS for L-methionine to be 0.12 ± 0.01 mM. This value is as low as those reported for this *E. coli* AdoMetS in previous studies, at 0.1 mM [7]. It is noteworthy that the present coupling assay is a nonradioactive continuous assay, whereas previous assays are discontinuous assays using radiolabelled methionine or ATP as a substrate to detect AdoMetS activity. These differences make this coupling assay more feasible and allow real-time monitoring of the reaction progress.

Linear standard curves of L-methionine ranging from 0-100 µM, 0-100 µM, and 0-10 µM were obtained when AdoMetS was coupled to the UV, colorimetric, and fluorescent assays, respectively (Fig. 4). Each assay took 5-10 minutes to complete the reaction after the preparation of the reaction mixtures. The slope of the standard curves for L-methionine was also similar to that for PPi, indicating that almost all L-methionine in the assay mixture was efficiently consumed to produce equimolar PPi. The detection limit of the colorimetric assay for L-methionine was estimated to be 1 µM. These results demonstrate that L-methionine can be accurately determined by the AdoMetS assay.

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**Table 1. Summary of the three kinds of assays used to detect PPi**

<table>
<thead>
<tr>
<th>Linear range of PPi concentrations (µM)</th>
<th>UV</th>
<th>Colorimetric</th>
<th>Fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-200</td>
<td>0-100</td>
<td>0-10</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Selectivity of the AdoMetS assay**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Met a</td>
<td>100</td>
</tr>
<tr>
<td>L-Ala, L-Cys, L-Asp, L-Glu, L-Phe, Gly, L-His, L-Ile, L-Lys, L-Leu, L-Asn, L-Pro, L-Gln, L-Fer, L-Thr, L-Val, L-Trp, and L-Tyr a</td>
<td>Not detected</td>
</tr>
<tr>
<td>D-Met b</td>
<td>0.6</td>
</tr>
</tbody>
</table>

a Activity was measured when present at 0.5 mM.
b Activity was measured when present at 5 mM.
c The activity toward L-methionine was taken as 100%.

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![Fig. 3. PPi determination in the presence of Pi, ATP, or human plasma in assay mixtures. Final concentrations of Pi, ATP, and plasma in the assay mixture are shown. Recovery rates were calculated by dividing the slope of each standard curve by that of the additive-free standard curve. The slopes of additive-free standard curves of the UV, colorimetric, and fluorescent assays were taken as 100%. Pi concentration ranges were 0-200, 0-100, and 0-10 µM for the UV, colorimetric, and fluorescent assays, respectively. Error bars indicate standard errors.](attachment:image.png)
Selectivity and robustness of the AdoMetS assay

To evaluate the selectivity of the AdoMetS assay, L-methionine was replaced in the assay mixture by another proteinaceous L-amino acid or glycine. Consequently, this coupling assay exhibited no significant activity toward the amino acids except L-methionine (Table 2), which demonstrates its higher selectivity than that of the conventional enzymatic assays for L-methionine. The AdoMetS assay showed slight reactivity toward D-methionine (Table 2) in accordance with a previous characterization [9]. The Km value of AdoMetS for D-methionine was estimated to be 25±2 mM by the present coupling assay. The low reactivity and high Km value for D-methionine suggest that D-methionine should not affect the determination of L-methionine by the AdoMetS assay.

The assay using methionine-γ-lyase is known to be sensitive to ammonium contamination as described above. On the other hand, the AdoMetS assay mixture contains ammonium as an activator for PPDK [15; 18], and no significant influence was observed when an extra amount of ammonium was added to the mixtures. Therefore, this assay is tolerant to ammonium contamination unlike the methionine-γ-lyase assay. It was also demonstrated that the addition of 10 mM urea had no significant effect on the assay as well.

Spike and recovery test with human plasma

Because L-methionine in the blood of homocystinuria patients often increases by more than 100 µM [19], the concentration of L-methionine is used as a biomarker for this disease. To verify whether the AdoMetS coupling assay was applicable to the blood test to detect the increase in L-methionine, 0-20 µM L-methionine was added to the assay mixtures containing 20% (v/v) human plasma, and the recovery rates were calculated. The estimated recovery rates were comparable to 100% with low intra- and inter-assay CVs (<7%) (Table 3). These results indicate that the increase in L-methionine comparable to that expected to be present in the blood of homocystinuric patients, which is known to be useful diagnostically, can be accurately and reproducibly detected by the AdoMetS assay. The assay estimated the intrinsic L-methionine concentration in the plasma sample was estimated to be 24 ± 3 µM. This value was consistent with that estimated by instrumental analysis, 26.9 µM, confirming the accuracy of the AdoMetS assay in biological samples.

Discussion

A selective and robust assay for L-methionine was developed in this study by coupling AdoMetS to the PPI-detection system. The AdoMetS coupling assay was embodied as three different kinds of detection methods, namely, UV, colorimetric, and fluorescent detection. This facilitates wider use of the coupling assay in various circumstances in which limited kinds of analytic instruments are available. The enzymes constituting the assay can be readily obtained by over-expression in E. coli (AdoMetS and PPDK) or by commercial purchase (LDH, POX, and HRP). The UV assay is the simplest among the three detection methods because it only requires LDH in addition to AdoMetS and PPDK as an enzyme in the assay mixture. The colorimetric and fluorescent assays are advantageous because of their low detection limits, and the latter is highly sensitive, especially to low concentrations of the substrate. The UV and colorimetric assays were shown to be tolerant to the presence of human plasma; thus, these two assays can be applied not only to biochemical studies, but also to the analysis of biological samples.

Many enzymatic assays require pretreatments such as the deproteination of analytes because some constituents in biological samples interfere with the assays [20]. In contrast, the AdoMetS assay allows easier and simpler measurements of L-methionine in plasma without any deproteination pretreatment. Since the contamination of thiol compounds and ammonia interferes with the conventional methionine-γ-lyase assay, insensitivity to these molecules provides an advantage of the AdoMetS coupling assay. Furthermore, the AdoMetS assay requires no pretreatment to remove branched amino acids, which have been shown to disturb the L-methionine determination

Table 3. Detection of L-Met increases in human plasma samples

<table>
<thead>
<tr>
<th>Added L-Met (µM)</th>
<th>Recovered L-Met (µM)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.7 (95%)</td>
<td>5.3</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td>10.2 (102%)</td>
<td>6.6</td>
<td>4.7</td>
</tr>
<tr>
<td>20</td>
<td>20.7 (103%)</td>
<td>3.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

a Final concentration in the assay mixtures.

b The recovered concentration was calculated by subtracting the value of the blank sample to which no L-Met was added. The recovery is shown in parentheses.

c The intra-assay CV was determined by assaying plasma samples in a single run (n=12).

d The inter-assay (between-run) CV was calculated from six independent runs.
by the methionine dehydrogenase assay in previous studies [4]. These features allow for the more selective and rapid measurement of L-methionine by the AdoMetS coupling assay than by conventional enzymatic assays.

L-Amino acid oxidase and dehydrogenase are typical enzymes applied to the quantification of amino acids because their activity can be easily detected using spectrophotometric methods [4; 14; 21; 22; 23]. These enzymes oxidize or dehydrogenate amino acids at the amino group, which is a common structure shared among all amino acids. Such an intrinsic feature of L-amino acid oxidase and dehydrogenase often causes broad substrate specificity, preventing these enzymes from being widely used for selective quantification [14]. In contrast to these enzymes, higher selectivity is expected for an enzyme that reacts with an amino acid at the side chain [20]. However, the side chains in proteinaceous amino acids often exhibit less reactivity than the amino group due to their high activation energies. Thus, some energy source such as ATP is sometimes necessary for enzymatic reactions at side chains. ATP-dependent enzymes advantageously catalyze various kinds of irreversible reactions with help from high-energy phosphate bonds in ATP as a driving force. The AdoMetS reaction, in which ATP and the side chain of L-methionine are involved, is one of the major factors providing the high irreversibility and selectivity to this coupling assay.

Although the PPI detection system was coupled to AdoMetS in this study, its coupling partner is not limited to AdoMetS and may be extended to many enzymes that generate PPI as a reaction product. The PPI assay developed here can be used as a versatile component to detect the activity of PPI-producing enzymes. Various methods have been employed to assay PPI-producing enzymes, such as a radioactive assay [7; 8], LC-MS/MS [24], and a colorimetric detection of Pi [25], and most of these methods are discontinuous assays carried out after termination of the reaction. The present PPI detection system allows rapid real-time monitoring of the activity of these enzymes as a continuous assay. Furthermore, the coupling systems of a PPI-producing enzyme and PPI detection are expected to be used as a selective and robust assay similar to the AdoMetS coupling assay shown in this study. New selective assays to quantify other biomolecules are currently being developed (M. Kameya and Y. Asano, manuscript in preparation). We believe that this approach will widen the strategy to develop a selective enzyme assay.

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L-Met assay based on pyrophosphate detection / M. Kameya et al.


