Quantitation of L-Amino Acids by Substrate Recycling between an Aminotransferase and a Dehydrogenase: Application to the Determination of L-Phenylalanine in Human Blood

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A spectrophotometric recycling assay for the quantitation of L-phenylalanine (and phenylpyruvate) has previously been reported (Cooper et al., Anal. Biochem. 183, 210–214, 1989). The procedure involves the coupling of bacterial phenylalanine dehydrogenase with rat kidney cytosolic glutamine transaminase K. The latter enzyme possesses high affinity for phenylpyruvate. Recycling results in a ≥50-fold increase in sensitivity over that of a conventional spectrophotometric “end point” analysis procedure. The spectrophotometric recycling procedure has now been adapted to the measurement of L-phenylalanine in microliter quantities of human blood. This procedure is 10 times more sensitive than provided by a commercial kit for the spectrophotometric measurement of L-phenylalanine in human blood. Moreover, the present results suggest that the recycling procedure adapted for fluorometry will be even more sensitive. By use of suitable dehydrogenases and amino acid aminotransferases it should be possible to quantitate amino acids (in addition to phenylalanine) in small quantities of human blood. © 1996 Academic Press, Inc.

For many metabolites it is possible to devise a recycling assay in which the coupling of two reactions (at least one of which is enzyme-catalyzed) results in an amplification and proportionate increase in sensitivity. For example, glutamate may be measured by an amplification (recycling) procedure involving coupling of aspartate aminotransferase with glutamate dehydrogenase (1). A similar strategy has been used for the measurement of L-phenylalanine (2). This amino acid has been quantitated by a recycling procedure in which purified Bacillus sphaericus phenylalanine dehydrogenase is coupled with cytosolic rat kidney glutamine transaminase K (Eqs. [1]–[3]) (2).

\[
\text{L-Phenylalanine} + \text{NAD}^+ + \text{H}_2\text{O} \leftrightarrow \text{phenylpyruvate} + \text{NADH} + \text{NH}_4^+ \quad [1]
\]

\[
\text{L-Glutamine} + \text{phenylpyruvate} \leftrightarrow \text{L-phenylalanine} + \alpha\text{-ketoglutaramate} \quad [2]
\]

Sum: \[
\text{L-Glutamine} + \text{NAD}^+ + \text{H}_2\text{O} \leftrightarrow \alpha\text{-ketoglutaramate} + \text{NADH} + \text{NH}_4^+ . \quad [3]
\]

The rate of oxidation of L-glutamine is directly proportional to L-phenylalanine (or phenylpyruvate) concentration. The reaction is monitored in a standard 1-cm cuvette by noting the rate of increase of absorption at 340 nm due to formation of NADH (\( \epsilon = 6.23 \times 10^3 \)). With appropriate levels of enzyme and incubation for 2 h it is possible to obtain an amplification factor of 50 (2). This amplification procedure was adapted to quantitate L-phenylalanine in rat tissues (2). By removing L-phenylalanine on a small cation exchange column it was also possible to measure small quantities of phenylpyruvate in the tissue extracts. The concentration of phenylpyruvate in rat tissues was found to be in the range of 2–5 \( \mu M \) (2). The equilibrium position of reaction 1 actually favors the reverse reaction (reductive amination, reaction to the left) except at very high pH values. However, the product of the glutamine

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transaminase reaction ($\alpha$-ketoglutarate) cyclizes to a lactam (>99%) ensuring that the overall reaction (Eq. [3]) is drawn far to the right.

An important concept in the design of cycling assays is that the combination of two enzymes should improve the selectivity toward the metabolite under investigation. For example, cytosolic glutamine transaminase K has a relatively broad specificity toward $\alpha$-keto acids, but it has a relatively high $V_{\text{max}}$ in the presence of phenylpyruvate. Moreover, the enzyme has an unusually low $K_m$ for phenylpyruvate (<20 $\mu$M) which is at least five times lower than that exhibited toward other $\alpha$-keto acids (3). Phenylalanine dehydrogenase exhibits some activity with tyrosine, methionine, and other amino acids, but has the highest activity with (and affinity for) L-phenylalanine among amino acids tested (4). Thus, although neither enzyme is completely specific for the L-phenylalanine/phenylpyruvate pair, by combining the two enzymes in a recycling assay the specificity toward L-phenylalanine is greatly improved.

We now report that the cytosolic glutamine transaminase K/phenylalanine dehydrogenase cycling assay can be used to quantitate L-phenylalanine in small ($\sim\mu$L) samples of human blood/serum.

**MATERIALS AND METHODS**

Glutamate transaminase K (6 units/mg) was purified from the cytosolic fraction of rat kidneys as described previously (5) and stored in 20% (v/v) glycerol containing 100 mM potassium phosphate buffer, pH 7.2, 4$^\circ$C. A unit of glutamate transaminase K activity is the amount of enzyme that catalyzes the formation of 1 $\mu$mol of phenylpyruvate per minute in a reaction mixture containing 20 mM L-phenylalanine, 5 mM $\alpha$-ketoglutarate and 100 mM ammonium buffer, pH 9.0, 37$^\circ$C. Phenylalanine dehydrogenase purified from Bacillus sphaericus (180 units/mg) (4) or from Bacillus badius (68 units/mg) (6) was stored in 30% (v/v) glycerol containing 20 mM potassium phosphate buffer, pH 7.2, and 10 mM 2-mercaptoethanol, 4$^\circ$C (2). A unit of L-phenylalanine dehydrogenase activity is the amount of enzyme that catalyzes the formation of 1 $\mu$mol of phenylpyruvate per minute in a reaction mixture containing 10 mM L-phenylalanine, 2.5 mM NAD$^+$, and 100 mM glycine-KOH buffer, pH 10.4, 25$^\circ$C. Amino acids, $\beta$-NAD$^+$, and NADH were obtained from Sigma Chemical Co. (St. Louis, MO). A fluorometric kit and a spectrophotometric kit for the quantitation of L-phenylalanine were obtained from Sapporo IDL (Sapporo City, Sapporo, Japan).

**RESULTS**

**Measurement of L-Phenylalanine in Human Blood by the Recycling Procedure**

Initial experiments were performed to determine whether the cytosolic glutamine transaminase K/phenylalanine dehydrogenase recycling procedure is applicable to well plate analyses. The original method of Cooper et al. (2) was used for the determinations except that B. badius phenylalanine dehydrogenase was used in place of the B. sphaericus enzyme and the absorbance change at 340 nm was monitored in a well plate analyzer at 25$^\circ$C. [The B. badius enzyme was found to be more suitable because it exhibits relatively less activity with L-tyrosine than does the B. sphaericus enzyme (6).] A linear response was obtained between rate of increase in absorbance at 340 nm over a 90-min period and phenylalanine concentration over the range of 0 to 750 pmol/100 $\mu$L assay volume (data not shown).

In a second series of experiments, a 3-mm-diameter piece was punched from filter paper that had been impregnated with 3 $\mu$L of normal human blood. Proteins in the sample were denatured on the disk by addition of 10 $\mu$L of a mixture of ethanol:acetone:distilled water (7:7:1; v/v/v). After drying at 37$^\circ$C for 30 min, phenylalanine in the sample was extracted (25$^\circ$C, 60 min) into 30 $\mu$L of deionized water and quantitated by the recycling assay of Cooper et al. (2) modified for well plate analysis. An aliquot of the extract (20 $\mu$L) was added to 80 $\mu$L of the enzyme recycling reagent containing 50 mM L-glutamate, 6.25 mM NAD$^+$, 250 mM glycine-KOH buffer, pH 10.4, 0.6 unit of B. badius phenylalanine dehydrogenase and 2.5 $\mu$L of rat kidney cytosolic glutamine transaminase K. After incubation at 25$^\circ$C for 90 min or 120 min, 800 $\mu$L of deionized water was added and the absorbance at 340 nm was recorded relative to a blank lacking L-phenylalanine. Figure 1 shows the increase in absorption at 340 nm for each of eight normal samples of human blood applied to filter paper (solid diamonds). L-Phenylalanine was added to these samples such that the final concentrations of this amino acid encompassed the range 10 to 2000 pmol/20 $\mu$L of extract (or 5 to 1000 pmol/$\mu$L of original blood sample). The values for increase in absorption over 90 min (A.A) at 340 nm due to recycling of phenylalanine in the blood samples (solid diamonds) were indistinguishable (except at very high concentrations) from those of comparable aqueous L-phenylalanine standards carried through the same procedure (solid squares). In another experiment, dialyzed human plasma samples were added to equal volumes of packed red blood cells previously loaded with known amounts of L-phenylalanine. Again, the values for the increase in absorbance at 340 nm due to known concentrations of phenylalanine in the red blood cells plus dialysate (open squares; Fig. 1) were similar to those of comparable aqueous L-phenylalanine standards.

Comparison of the Performance of the Recycling Assay for L-Phenylalanine with That of Commercial Kits

Two kits have been developed by Sapporo IDL for the measurement of L-phenylalanine in human blood.
and are commercially available. The first (fluorometric) kit relies on a microplate fluorometer and is based on the direct fluorometric determination of NADH generated from the oxidation of L-phenylalanine in the phenylalanine dehydrogenase reaction (Eq. [1]) (excitation at 340 nm; emission at 450 nm). The second (spectrophotometric) kit is based on the method of Naruse et al. (7). NADH formed from the oxidation of L-phenylalanine in the L-phenylalanine dehydrogenase-catalyzed reaction reduces Co$^{3+}$ to Co$^{2+}$ in the presence of the electron carrier 1-methoxy-5-methylphenazium methysulfate. The generated Co$^{2+}$ forms a highly colored chelate in the presence of 2-(5-bromo-2-pyridazono)-5-N-propyl-N-sulfopropylaminophenol which absorbs strongly at 590 nm (7).

Known amounts of L-phenylalanine in 20 μl of deionized water (for the recycling assays) or in 50 μl of 0.3 M trichloroacetic acid (for the kit assays) were subjected to separate well plate assay procedures: (1) spectrophotometric recycling assay, (2) recycling assay as in 1 but analyzed in the fluorometric mode, (3) spectrophotometric kit assay, and (4) fluorometric kit assay (Table 1). The data show that the signal obtained with the spectrophotometric recycling assay is 10 times greater than that obtained with the spectrophotometric kit assay (Table 1, column 2 versus column 4). However, the response of the recycling assay, but not the kit assay, to high concentrations of phenylalanine is non-linear. The nonlinear response of the recycling assay at high concentrations of phenylalanine is due to insufficient activity of coupling enzymes. Therefore, for analysis of L-phenylalanine by the recycling procedure the level of this amino acid must be maintained below 1.5 nmol within the assay mix. Table 1 shows that the signal obtained from the fluorescence recycling procedure is ~5 times greater than that obtained from the commercial fluorescence assay kit. The blank value is greater in the former case and adds to the inaccuracy of the determination. However, no attempt was made in the present work to minimize the blank fluorescence.

**DISCUSSION**

Several bacterial phenylalanine dehydrogenases have been characterized within the past 10 years (e.g., Refs. 4, 6, 8, 9) and Hummel et al. were the first to suggest that these enzymes could be used for the measurement of L-phenylalanine in human neonatal blood (8). The original procedure required measurement of initial reaction velocities in the presence of L-phenylal-
TABLE 1

<table>
<thead>
<tr>
<th>L-Phenylalanine (pmol)</th>
<th>Spectrophotometric recycling assay$^a$</th>
<th>Fluorometric recycling assay$^c$</th>
<th>Spectrophotometric kit assay$^d$</th>
<th>Fluorometric kit assay$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Blank (0))</td>
<td>(0.152)</td>
<td>(785)</td>
<td>(0.090)</td>
<td>(60)</td>
</tr>
<tr>
<td>36</td>
<td>0.009</td>
<td>63</td>
<td>0.001</td>
<td>9</td>
</tr>
<tr>
<td>50</td>
<td>0.017</td>
<td>97</td>
<td>0.002</td>
<td>12</td>
</tr>
<tr>
<td>750</td>
<td>0.267</td>
<td>842</td>
<td>0.023</td>
<td>174</td>
</tr>
<tr>
<td>3600</td>
<td>0.401</td>
<td>1327</td>
<td>0.113</td>
<td>867</td>
</tr>
</tbody>
</table>

$^a$ The values obtained for each concentration of phenylalanine have been corrected by subtraction of the blank.
$^b$ $\Delta$ absorbance at 340 nm/90 min.
$^c$ $\Delta$ fluorescence/90 min in arbitrary units.
$^d$ $\Delta$ absorbance at 590 nm (independent of time).
$^e$ $\Delta$ fluorescence in arbitrary units (independent of time).

The procedure, however, was not sensitive. Later, Hummel and colleagues improved the sensitivity by devising a microplate assay (10) and a spectrophotometric end-point assay (11). By coupling the phenylalanine dehydrogenase reaction with cytosolic glutamine transaminase K, we showed that it was possible to increase the sensitivity of detection of phenylalanine at least 50-fold over that of spectrophotometric end-point procedures (4). In the present work we have extended these findings further and shown that it is possible to use the recycling assay and well plate analyses to measure L-phenylalanine in small (µl) amounts of human blood. The procedure is capable of measuring phenylalanine in the low normal range (i.e., 25 µM, corresponding to 50 pmol in the assay mixture (Table 1; column 2)). Such low levels are difficult to quantitate by end-point spectrophotometric analysis because of very small changes in absorbance (Table 1, column 4). Indeed, the sensitivity of the spectrophotometric recycling procedure is presently 10-fold greater than that of a commercial spectrophotometric kit and promises to be even more sensitive when adapted to fluorometry.

It is possible that recycling assays involving transaminases coupled to dehydrogenases can be devised to amplify other amino acids in addition to L-phenylalanine and L-glutamate. For example, aminotransferases and dehydrogenases that are relatively specific for branched-chain amino acids have been well characterized. Therefore, it should be possible to devise a recycling assay for branched-chain amino acids that might be useful in the screening of human blood for abnormally high levels of these amino acids. Mammalian tyrosine aminotransferase has a much higher affinity for L-tyrosine than for L-phenylalanine (12). B. sphaericus phenylalanine dehydrogenase is also an effective L-tyrosine dehydrogenase (4). If a more specific bacterial tyrosine dehydrogenase could be found then this would permit the development of a recycling assay for L-tyrosine involving this enzyme and mammalian tyrosine aminotransferase. Similarly, if appropriate transaminases and dehydrogenases can be discovered, then recycling assays could perhaps be devised for the measurement of homocysteine (after trapping of the sulphydryl with a suitable reagent) and of methionine.

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REFERENCES