METABOLISM OF AROMATIC AMINO ACIDS BY THE RAT HEART AND DIAPHRAGM

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1. Introduction

Because incubation of [U-^{14}C]phenylalanine or tyrosine with muscle preparations does not produce $^{14}CO_2$, it is assumed that these aromatic amino acids are poorly metabolised [1–3]. Release of aromatic amino acids from muscle preparations in the presence of cycloheximide has therefore been used as an index of muscle protein degradation. However, there is some evidence that aromatic amino acids may be at least transaminated by muscle [4,5]. This might interfere with methods of detection which are dependent upon the presence of an amino group, e.g., amino acid analysis or the amino acyl tRNA synthetase method [6]. Here, the transamination of aromatic amino acids by homogenates and whole tissue preparations is reported.

2. Experimental

2.1. *Materials*

Radiochemicals were from The Radiochemical Centre, Amersham HP7 9LL. Biochemicals and other chemicals were either from Sigma (London) Chemical Co., Poole BH17 7NH, or BDH Chemicals, Poole BH12 4NN.

2.2. *Homogenization*

Rats were killed by a blow on the head and cervical dislocation. Hearts were removed, rinsed in 0.9% (w/v) NaCl, frozen in liquid N_2 then homogenized at 0°C in 50 mM potassium phosphate/0.1 mM pyridoxal phosphate (pH 6.8) (1 g tissue + 4 vol. buffer) using a Polytron homogenizer (Kinematica Gmbh., CH-6005 Luzern) at mark 10 until the tissue was completely dispersed. Homogenates were centrifuged at 150 000 × *g* for 5 min and supernatants (sometimes dialyzed overnight at 4°C against homogenization buffer) were used in experiments.

2.3. *Radiochemical assay of aminotransferase activity*

In pyridoxal phosphate-dependent transamination, a proton from the 2-position of the amino acid is lost to water [7,8]. If the 2-hydrogen is replaced by a tritium atom, ^3H_2O formed during transamination can be easily separated from the amino acid by ion-exchange chromatography. There are problems with this approach (exchange reactions, racemases or L-amino acid oxidase also remove the 2-proton, only [2,3-^{3}H] amino acids commercially available). These can be circumvented if it can be shown that: (i) the appearance of ^3H_2O is dependent on the presence of an amino-group accepting 2-oxo acid; (ii) that any 2-oxo [3-^{3}H] acid product can be quantitatively separated from ^3H_2O or [2,3-^{3}H]amino acid substrate.

Alanine aminotransferase (EC 2.6.1.2) was assayed at 30°C in 0.4 ml containing 50 mM potassium phosphate (pH 6.8)/0.1 mM pyridoxal phosphate/10 mM 2-oxoglutarate (pH 7 with KOH)/37.5 mM L-[2,3-^{3}H]-alanine (spec. radioact. 1 μCi/μmol) and 4 μg enzyme (Sigma lot 69C-9841 diluted in 90 mM potassium phosphate (pH 6.8)/0.2 mM pyridoxal phosphate/1 mg bovine serum albumin/ml). A 50 μl sample was removed at various times and 25 μl 1 M HCl added. A sample (50–70 μl) of terminated reaction mixture was transferred to a column (0.5 × 3 cm) of Dowex 50W × 4 (H^+ form) precycled as in [9]. An initial water (0.5 ml) wash was discarded, products were eluted with two 0.5 ml water washes into scintillation vials and counted in toluene–methoxyethanol based scintillant [10].

Phenylalanine aminotransferase activity was estimated similarly except that L-phenyl [2,3-^{3}H]alanine (section 3 for concentrations) replaced alanine and

rat heart supernatant replaced alanine aminotransferase.

For both assays:

(i) ^3H_2O and 2-oxo acid product were stoicheiometrically recovered;
(ii) 98–99% L-[2,3-^{3}H]amino acid was retained by the column;
(iii) Appearance of radioactivity above the blank values was dependent upon the presence of 2-oxoglutarate;
(iv) The time course was linear for at least 10 min;
(v) The rate of product appearance was linear with enzyme added.

2.4. *Tissue preparations*

For the isolated diaphragm, male fed rats (80–100 g) were killed by a blow on the head and decapitation. Diaphragms were removed into Krebs-Henseleit bicarbonate solution [11] at 25°C equilibrated with 95% O_2/5% CO_2 in the absence or presence of glucose (section 3). Diaphragms were cut into halves or quarters, blotted lightly and weighed into 25 ml Erlenmeyer flasks or scintillation vials containing 2–3 ml Krebs-Henseleit bicarbonate solution with additions described in section 3. Diaphragms were preincubated in a shaking water bath (60 cycles/min) at 37°C for 30 min and were then placed in fresh media for the 2 h test incubation. During this time, the media were deproteinized by the addition of perchloric acid (0.05 vol.) and centrifugation, or metabolism stopped by addition of 0.5 vol. 1 M HCl.

Heart perfusions were carried out essentially as in [12]. Male fed rats (300–400 g) were killed by the intraperitoneal injection of 1 ml Sagatal (May and Baker, Dagenham, Essex) and hearts removed into ice-cold 0.9% (w/v) NaCl. Hearts were perfused using recirculation of 15 ml medium (+10 mM glucose) after an initial wash-through of 15 ml medium. L-Phenyl [2,3-^{3}H]alanine (0.1 mM, 1 μCi/ml) was added to the medium after the wash-through. Samples (50 μl) were analyzed for [^{3}H]products as in section 2.3. Samples (10 μl) were removed from the terminated extracts for the determination of total radioactivity.

2.5. *Other methods*

Alanine aminotransferase was assayed spectrophotometrically as in [13] at 37.5 mM L-alanine and pH 6.8. The reaction was initiated by the addition of 0.25–0.75 μg enzyme diluted as above. (1 unit = 1 μmol substrate used/min at 30°C). Alanine in $HClO_4$-treated media (adjusted to pH 10 with KOH and stood at 0°C for 1 h) was assayed as in [14] at pH 8.5 using alanine dehydrogenase dialyzed against 5 mM EDTA (pH 7.5). Blank incubations used $HClO_4$-treated Krebs-Henseleit buffer containing appropriate concentrations of aromatic amino acids.

3. Results

3.1. *Validity of the radiochemical method of measurement of aminotransferase activity*

Assays for alanine aminotransferase were carried out spectrophotometrically and radiochemically by construction of a plot of enzyme activity versus enzyme added (μg). From the slope (not shown), the specific activity was calculated. The radiochemical assays gave an activity of 26.9 units/mg enzyme at 30°C and the spectrophotometric method gave 26.3 units/mg enzyme, proving the validity of the radiochemical method and showing (with the controls in section 2.3) that it measures flux through the reaction rather than an isotope exchange of the part reaction. The rate of product appearance was linear with enzyme concentration to 10 μg protein /ml.

3.2. *Phenylalanine transamination by rat heart supernatants*

Incubation of rat heart supernatants with L-phenyl [2,3-^{3}H]alanine produced radioactivity which was not retained by the cationic ion-exchange column. Control experiments (section 2.3) suggest that this is consistent with phenylalanine being transaminated to form glutamate, phenyl [3-^{3}H]pyruvate and ^3H_2O. The nature of the products was not well characterized but under the conditions of this initial rate study, it is presumed that tritium will be present in phenylpyruvate rather than any more extensively metabolised product (if this can occur at all). The V_{max} and app. K_m of phenylalanine aminotransferase for phenylalanine (fig.1) were 12.23 ± 0.13 μmol . 30 min^{-1} . g fresh wt $heart^{-1}$ and 20.3 ± 0.6 mM, respectively (10 mM 2-oxoglutarate, 30°C, results as means ± SEM for n = 3). Aminooxyacetate (an inhibitor of pyridoxal phosphate-dependent enzymes, see [15]) inhibited phenylalanine transamination (fig.2). Neither commercially-available pig heart alanine aminotransferase nor aspartate aminotransferase (EC 2.6.1.1) catalysed phenylalanine transamination (not shown).

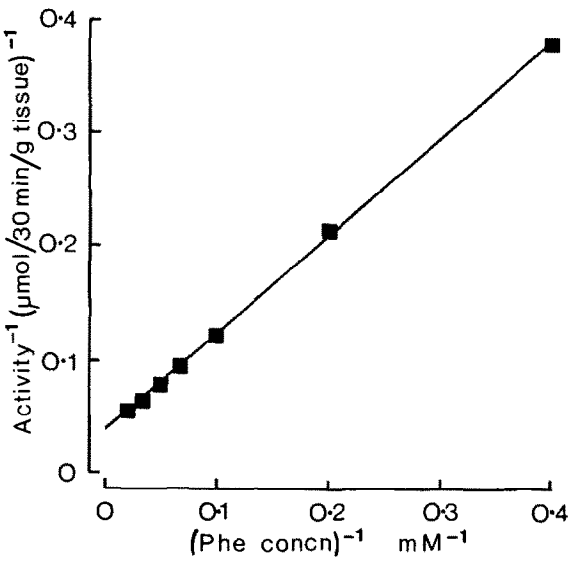

Fig.1. Reciprocal plot for phenylalanine aminotransferase of rat heart supernatant fraction. Incubations were at 30°C for 30 min. The incubation volume was 100 μl and contained 20 mM potassium phosphate (pH 6.8)/0.1 mM pyridoxal phosphate/10 mM 2-oxoglutarate (neutralized)/0.5 μCi L-phenyl [2,3-^{3}H]alanine plus various amounts of L-phenylalanine. The reaction was initiated by addition of rat heart supernatant fraction (40 μl) prepared as in section 2.2. It was terminated by the addition of 1 M HCl (50 μl) and products separated as in section 2.3. Results were plotted by the Lineweaver-Burk method [20].

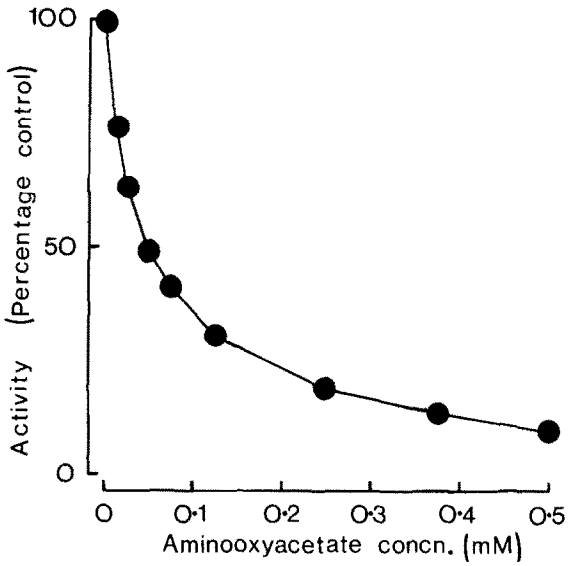

Fig.2. Inhibition of phenylalanine transaminase by aminooxyacetate. Incubations were as described in fig.1 except that L-phenylalanine was 1 mM and the incubations also contained various concentrations of aminooxyacetate (neutralized to pH 7 with KOH). Phenylalanine transaminase activity is expressed as the percentage of control activity in the absence of aminooxyacetate.

3.3. *Metabolism of L-phenyl [2,3-^{3}H]alanine by the perfused rat heart and the isolated rat diaphragm*

Perfusion of rat hearts with media containing 0.1 mM L-phenyl [2,3-^{3}H]alanine produced radioactivity which was not retained by cationic ion-exchange columns (fig.3a). This was presumably ^3H_2O and phenyl [3-^{3}H]pyruvate. The phenylalanine concentration in the perfusate was approximately the same as in rat plasma [16]. Metabolism of phenylalanine was inhibited by 1 mM aminooxyacetate (fig.3a). Since the products of transamination of phenylalanine in the perfusate were not well characterized and since phenylalanine is released from the heart during perfusion, an accurate estimate of the rate of phenylalanine transamination is not possible. Thus, results are expressed in terms of [^{3}H]product produced in ng atom/g dry wt heart based upon the original specific radioactivity. However, by making certain assumptions, the rate of phenylalanine transamination can be calculated and can be compared to the rate of transamination in heart extracts. The following assumptions were made:

(i) Phenyl [3-^{3}H]pyruvate was not metabolised further and is released into the perfusate;
(ii) The enzyme activity doubled for an 8°C rise in temperature;
(iii) The contribution of endogenous phenylalanine was small.

(The calculated contribution of endogenous phenylalanine to the perfusate phenylalanine was only ~10%.) From the data in fig.3a, the rate of transamination of phenylalanine by the perfused heart was 0.025 μmol . 30 min^{-1} . g fresh wt^{-1} at 0.1 mM phenylalanine. This compares with a computed rate of 0.125 μmol . 30 min^{-1} . g fresh wt^{-1} using data in section 3.2.

The isolated rat diaphragm also metabolised phenylalanine (fig.3b). The appearance of [^{3}H]product in the incubation medium was linear with time for ⩾2 h. The rate of phenylalanine transamination was linear over 0.1–1 mM phenylalanine. This is presumably a consequence of the relatively high app. K_m of the aminotransferase for phenylalanine.

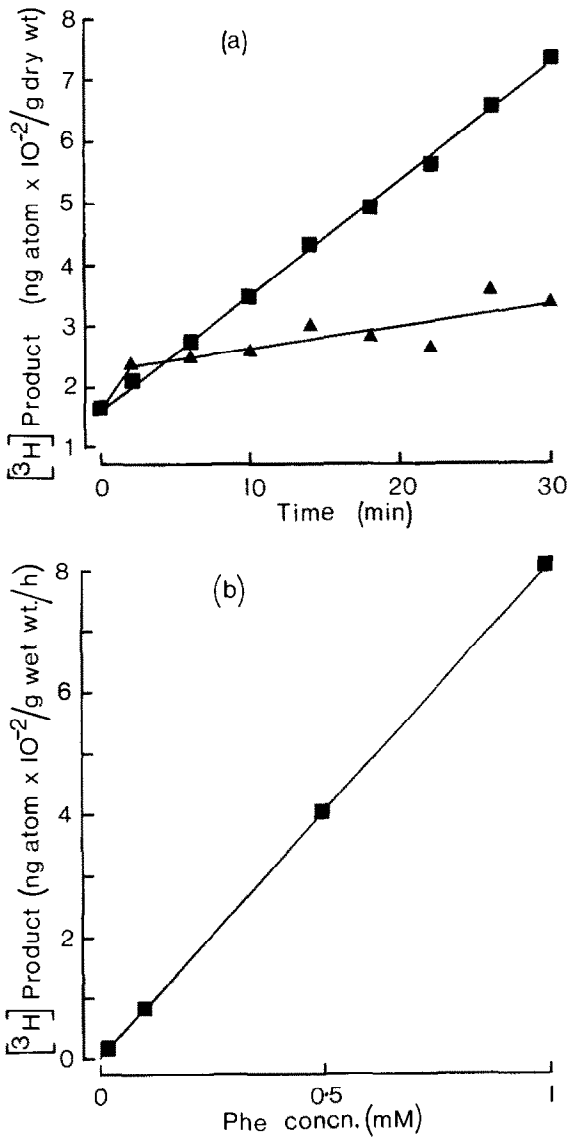

Fig.3. Metabolism of phenylalanine by the perfused rat heart and isolated rat diaphragm. Tissues were incubated as in section 2.4. In (a) the transamination of L-phenyl [2,3-^{3}H]-alanine (0.1 mM) by the perfused heart is shown (■). The inhibition of L-phenyl [2,3-^{3}H]alanine (0.1 mM) transamination by 1 mM aminooxyacetate is also shown (▲). In (b) the concentration dependence of L-phenyl [2,3-^{3}H]alanine transamination by the isolated hemidiaphragm is shown. Diaphragm incubations were in 2 ml Krebs-Henseleit medium [11] containing half the normal calcium concentration and supplemented with 10 mM glucose and 0.5 mM cycloheximide. After 30 min preincubation, diaphragms were transferred to fresh media containg 10 μCi L-phenyl [2,3-^{3}H]alanine and carrier L-phenylalanine as indicated, in addition to the additions in the preincubation. Samples (50 μl) were withdrawn at 30 min intervals and 25 μl 1 M HCl added. Products were separated from phenylalanine as in section 2.3.

3.4. *Stimulation of alanine release from the isolated diaphragm by aromatic amino acids*

Appearance of [^{3}H]product from phenylalanine metabolism does not demonstrate that there is any net flux through the phenylalanine transamination reaction. It could result from an isotope exchange reaction. Both phenylalanine and tyrosine stimulated alanine formation in the isolated diaphragm. Rates of alanine formation ± SEM (nmol . 2 h^{-1} . g fresh wt^{-1}) were:

Control 2.20 ± 0.14 (n = 6), +3.3 mM phenylalanine 2.64 ± 0.13 (n = 6);

Control 1.98 ± 0.22 (n = 8), +3.3 mM tyrosine 2.63 ± 0.16 (n = 11);

+10 mM glucose 3.05 ± 0.08 (n = 5), +10 mM glucose and 3.33 mM phenylalanine 3.74 ± 0.15 (n = 5).

Rates of alanine formation for + amino acids vs – amino acid incubations were significant at at least $p \leqslant 0.05$ (Student's t-test). This shows there is a net flux through aromatic amino acid transamination. Aromatic amino acids cannot form citric acid cycle intermediates in the diaphragm since [U-^{14}C]aromatic amino acids do not produce $^{14}CO_2$ [2]. Formation of alanine presumably proceeds via transamination of glycolytically produced pyruvate, either directly or via increasing the amino group pool available for transamination, rather than by providing carbon for de novo synthesis of pyruvate via the phosphoenol pyruvate carboxykinase reaction [17]. Because concentrations of aromatic amino acids used in these experiments were ~30-times plasma concentrations, there must be doubt about the physiological significance of alanine formation under these conditions.

4. Discussion

Both the heart and diaphragm can transaminate aromatic amino acids. They possess a phenylalanine aminotransferase distinct from alanine or aspartate aminotransferase. This casts doubt on the use of aromatic amino acid release in the presence of cycloheximide as a measure of muscle proteolysis. Substances which increase transaminatable 2-oxo acid concentrations might therefore decrease aromatic amino acid release. The enzyme(s) responsible for aromatic amino acid transamination is not known. In rat liver, there is a tyrosine–2-oxoglutarate aminotransferase (EC 2.6.1.5) which also poorly transaminates phenylalanine [18] and a phenylalanine–pyru-

vate aminotransferase [19]. This report also shows that high concentrations of aromatic amino acid stimulate alanine release from the diaphragm and provide an enzymological basis for this.

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