Metabolic Fates of L-Tryptophan in *Saccharomyces uvarum* (*Saccharomyces carlsbergensis*)

Mariko SHIN, Tetsuro SHINGUU, Keiji SANO and Chisae UMEZAWA*

School of Pharmacy, Kobe-Gakuin University, Nishi-ku, Kobe 651-21, Japan. Received December 14, 1990

The metabolism of L-tryptophan by *Saccharomyces uvarum* (*carlsbergensis*) was investigated by simultaneous measuring of fluxes through kynureninase, through transaminases and into protein using L-[methylene- ^{14}C]- and L-[side chain-2,3- ^3H]tryptophan. In yeast cultivated in synthetic medium (S medium), the flux into protein was predominant, closely followed by the flux leading to 2- ^3H liberation. The proportion of L-tryptophan metabolized via the latter flux increased over 10-fold (75% of total tryptophan metabolized) as the concentration of L-tryptophan was raised from 5×10^{-5} to 5×10^{-4} M. L-Tryptophan metabolized via the kynureninase flux was less than 5% of total tryptophan metabolized. In yeast extract-polypepton-glucose medium (YPG medium), more tryptophan was incorporated into protein than in the S medium. Contribution of the kynureninase flux remained very low. Tryptophan metabolism via each flux changed depending on the growth phase. 2- ^3H liberation was shown to be primarily due to tryptophol synthesis by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR), indole-3-acetic acid and kynurenic acid also contributing to 2- ^3H liberation but to a much lesser extent. 2- ^3H liberation increased dose-dependently at tryptophan concentration higher than 10^{-5} M, while the kynureninase flux reached its plateau at 10^{-5} M. Formation of tryptophol and indole-3-acetic acid via indole-3-pyruvic acid and indole-3-acetaldehyde with indole aldehyde as a by-product was confirmed using exogenous tryptophan metabolites with indole rings.

Keywords *Saccharomyces uvarum* (*carlsbergensis*); L-tryptophan; metabolic flux; charcoal non-adsorbed metabolite; kynureninase; transaminase; tryptophol

Tryptophan is known to be metabolized by a variety of pathways and to supply physiologically important substances. In *Saccharomyces*, tryptophan was postulated to be catabolized via indole-3-pyruvic acid (IPA) to tryptophol (TOH),¹⁻⁴⁾ which was later determined to be the main degradation product of tryptophan.^{5,6)} The degradation to anthranilic acid (AA)⁷⁾ and 3-hydroxy anthranilic acid^{8,9)} was postulated, and in aerobically grown *Saccharomyces cerevisiae* biosynthesis of nicotinic acid from tryptophan was also reported.¹⁰⁾ However, no studies have ever been done on the contribution rate of each metabolic pathway to total tryptophan metabolism in *Saccharomyces*.

Smith *et al.* examined tryptophan metabolism in rat hepatocytes by measuring charcoal-non-adsorptive radioisotope release from several radioisomers of tryptophan.¹¹⁾ Kiyohara *et al.* reconfirmed the usefulness of this method in studying tryptophan metabolism in rat liver cells.¹²⁾ In the present study, we applied the method for estimating metabolic fluxes of tryptophan in yeast with several modifications and were able to elucidate the flux via each metabolic pathway simultaneously under several different conditions.

Experimental

Organism, Media, and Growth Conditions *Saccharomyces uvarum* (*Saccharomyces carlsbergensis*) ATCC 9080 was used throughout this work. In most studies, cultures were grown in S medium (glucose, 20 g; KH_2PO_4 , 3 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 g; inositol, 10 mg; D-biotin, 2.2 μg ; Ca-pantothenate, 1 mg; thiamine-HCl, 1 mg; pyridoxine-HCl, 1 mg per liter, pH 4.5).¹³⁾ YPG medium (yeast extract, 3.5 g; polypepton, 3.5 g; KH_2PO_4 , 2.0 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; MgSO_4 , 1.0 g; glucose, 10 g per liter, pH 6.2) was also used on some occasions. The yeast was cultivated aerobically in 1 l Meyer flasks containing 250 ml of medium at 30°C at 110 oscillations min^{-1} . Cells in mid-logarithmic growth phase were washed and resuspended with fresh medium to a final density of 2.5 mg dry weight (wt.) cells/ml.

Chemicals Tryptophan, kynurenine (KN) sulphate, kynurenic acid (KA), xanthurenic acid (XA), *p*-phenylethylamine and charcoal Norit A were obtained from Nacalai Tesque Inc. Indole-3-acetaldehyde was purchased from Sigma, indole aldehyde (IAld) from Fluka AG Chem, indole-3-acetic acid (IAA) from Wako Pure Chemicals and TOH from

Tokyo Kasei Co., Ltd. Scintillation cocktail ACS II and Soluene-350 were obtained from Amersham International and Packard Instruments, respectively. L-[Methylene- ^{14}C]tryptophan (specific radioactivity 59 Ci/mol) and L-[side chain 2,3- ^3H]tryptophan (specific radioactivity 62 Ci/mmol) were obtained from Amersham International and Commissariat à l'Énergie Atomique, respectively. Each radiolabelled tryptophan was further purified by high performance liquid chromatography (HPLC).

Measurement of Liberated $^{14}\text{CO}_2$ and Charcoal Non-adsorbed Metabolites from Radioisomers of Tryptophan The method of Smith *et al.*¹¹⁾ was used with modifications as follows: Portions (0.25 ml) of cell suspension (2.5 mg dry wt. ml^{-1}) were bubbled with O_2 in a test tube (1.6 $\times$ 10.4 cm). The tubes were capped with rubber stoppers with suspended center wells. Incubations were started by the injection of 50 μl of radiolabelled tryptophan solution through the rubber stopper by a syringe, and carried out under an atmosphere of O_2 at 30°C, at 110 oscillations min^{-1} . Incubations were terminated by the injection of 0.1 ml of 2.2 M-HClO₄. *p*-Phenylethylamine-methanol (1:1, 0.2 ml) was injected into the center well, which contained a piece of folded filter paper. Both additions were made through a septum in the stopper. All test tubes were then shaken at 75 oscillations min^{-1} at room temperature for a further 30 min, to ensure complete absorption of released CO_2 . The filter paper, with 500 μl of methanol washing, was transferred to a vial containing 10 ml of ACS II for counting radioactivity.

Portions of the supernatant after centrifuging the HClO₄-treated reaction mixture were analyzed for charcoal non-adsorbed metabolites. Two hundred μl of cell-extract was treated with 60 μl of charcoal suspension (50 mg Norit A in 1 ml water). After thorough mixing and 30 min of standing, the charcoal was removed by centrifugation (13000 $\times g$, 3 min) and charcoal non-adsorbed radioactivity in 180 μl of the supernatant was counted in ACS II. Residual tryptophan in the acidic supernatant was assayed by the method of Denckla and Dewey.¹⁴⁾

Assay Method of Incorporation of Tryptophan into Protein A modification of the filter disc method of Mans and Novelli was employed.¹⁵⁾ Twenty or 40 μl of the HClO₄-treated samples incubated with either L-[side chain 2,3- ^3H]- or L-[methylene- ^{14}C]tryptophan was filtered through glass fiber filters (Advantec Toyo, GC50, i.d. 24 mm), and the residue was washed with 5 ml of 0.5 M HClO₄ followed by the same amount of ethanol. The washed filters were transferred to scintillation vials containing 0.5 ml of Soluene, 0.2 ml of 1 M-HCl and 10 ml of ACS II to count radioactivity.

Identification and Determination of Tryptophan Metabolites The yeast was cultivated aerobically in S medium with 5×10^{-4} M non-labelled tryptophan for 3 h. The major metabolite was extracted with acidic ethyl acetate from the broth. The concentrated extract was dissolved in methanol and subjected to HPLC [column, Finepak SIL C₁₈₋₅, 4.6 mm $\times$ 250 mm; mobile phase, 10^{-2} M phosphate buffer (pH 4.0)-methanol-acetonitrile

sum of the radioactivity of $^{14}\text{CO}_2$ and ^{14}C -labelled alanine. A preliminary experiment showed that L-[methylene- ^{14}C]-tryptophan was completely ($98.9 \pm 0.26\%$) adsorbed by charcoal, while L-[1- ^{14}C]alanine was not adsorbed ($1.25 \pm 0.15\%$). Tryptophan metabolized *via* the kynureninase flux was calculated from the equation:

On the other hand, radioisotope liberation from L-[side chain 2,3-³H]tryptophan may occur at several stages of tryptophan metabolism by the following reactions: (1) L-[2,3-³H]Alanine formation by the action of kynureninase or 3-hydroxy kynureninase; (2) 2-³H liberation by the action of transaminases on tryptophan, KN or 3-hydroxy-kynurenine (OHKN). Therefore, total radioisotope release from L-[side chain-2,3-³H]tryptophan may represent the sum of tryptophan metabolized *via* the kynureninase flux, and that metabolized *via* KN- or OHKN-transaminase (EC 2.6.1.7) and tryptophan transaminase (EC 2.6.1.27). As only one ³H out of 3 is liberated, the transaminase flux (2-³H liberation) was calculated from the equation:

$$[(^3\text{H non charcoal-adsorbed dpm}) - (\text{alanine formed nmol})]$$

Chart 1 outlines the possible steps at which radiolabelled moieties may be released from L-[methylene- ^{14}C]tryptophan or L-[side chain-2,3- ^3H]tryptophan.

^{14}C from L-[methylene- ^{14}C]tryptophan can be released only *via* the kynureninase pathway, and then L-[^{14}C]alanine formed may be oxidized to $^{14}\text{CO}_2$. Therefore, a flux of tryptophan through kynureninase was estimated by the

Outline of L-tryptophan expected in the yeast is shown. Radioisotope nomenclature: ○, L-[methylene-¹⁴C]tryptophan; ●, L-[side chain-2,3-³H]tryptophan. The metabolic fates: (1), alanine liberation (kynureninase flux); (2), 2-H liberation (transaminase flux).

$$\times (\text{specific radioactivity of L-[2,3-}^3\text{H]tryptophan}) \\ \div [(\text{specific radioactivity of L-[2,3-}^3\text{H]tryptophan})/3]$$

A possibility that the metabolic flux of tryptophan to protein is overestimated by the simultaneous counting of the incorporation of ^{14}C -alanine formed from L-[methylene- ^{14}C]tryptophan by kynureninase was checked. Pulse labelled protein was hydrolyzed with alkali and the hydrolysate was subjected to paper chromatography. The chromatograms (data not shown) showed that more than 90% of radioactivity found in the protein fraction was retained in tryptophan, which indicates that any L-[^{14}C]alanine secondarily formed from L-[methylene- ^{14}C]tryptophan was hardly incorporated into protein. This is probably due to the fact that the intracellular alanine pool is far bigger than that of tryptophan.¹⁸⁾

Metabolism of 5×10^{-5} or 5×10^{-4} M tryptophan by yeast cultivated in S medium is shown in Fig. 1. In 5×10^{-5} M tryptophan (A), the flux into protein was predominant (40–50%) closely followed by the flux into $2\text{-}^3\text{H}$ liberation (40%). During 3 h-incubation, tryptophan

Fig. 1. Time Course of the Main Fluxes of L-Tryptophan Metabolism

Yeasts were cultivated in S medium with 5×10^{-5} M (A) or 5×10^{-4} M (B) tryptophan in a test tube containing 300 μl of broth. $\square$, acid insoluble fraction; $\boxtimes$, 2-H liberation; $\blacksquare$, alanine liberation; $\square$, unidentified.

Fig. 2. Tryptophan Metabolism via Each Flux Throughout the Growth Phase

Yeasts cultivated in YPG medium were reincubated with radiolabelled tryptophan in a test tube containing 300 μl medium (150 nmol tryptophan added) for 1 h. $\text{---}\blacktriangle\text{---}$, growth; $\text{---}\bullet\text{---}$, alanine liberation; $\text{---}\circ\text{---}$, 2-H liberation; $\text{---}\triangle\text{---}$, flux into protein.

incorporated into the acid-insoluble fraction and metabolized via the $2\text{-}^3\text{H}$ liberating reactions increased significantly. The kynureninase flux (alanine liberation) was very small (2–4%) throughout the entire incubation period. When the initial concentration of tryptophan was raised to 5×10^{-4} M (B), the flux leading to $2\text{-}^3\text{H}$ liberation increased 10 times (75%), the flux into the acid insoluble fraction and the flux through kynureninase also increased but to lesser extent.

In YPG medium, 30–40% of added tryptophan (5×10^{-4} M) was incorporated into the acid-insoluble fraction, the figure being much higher than that obtained in S medium. This is because in YPG medium, other amino acids exist in abundance and tryptophan may be a rate limiting factor for protein synthesis. The 2-H liberation flux increased with time, while the kynureninase flux remained small ($2.5 \pm 0.9\%$ of total tryptophan metabolized) during 3 h-incubation.

Metabolic fluxes of tryptophan in different growth phases were investigated by periodic collection of cultured broth in YPG medium. As shown in Fig. 2, the amount of tryptophan incorporated into protein was parallel to the cell growth, and at the stationary phase the incorporation ceased. On the other hand, the flux via alanine liberation was increased in the logarithmic phase, then reduced at the

TABLE I. Comparison of the Amount of Tryptophan Metabolized via Side Chain 2-H Liberation with the Amounts of Metabolites Formed Following 2-H Liberation

Metabolites	Added tryptophan (nmol in test tube)			
	5×10^{-5} M (15 nmol)		5×10^{-4} M (150 nmol)	
	nmol	%	nmol	%
Metabolized tryptophan	12.7 ± 0.76	100	75.2 ± 7.76	100
Side chain 2-H liberation	6.09 ± 0.90	47.9	52.5 ± 4.82	69.9
KA	0.25 ± 0.05	2.1	0.97 ± 0.16	1.3
TOH	4.70 ± 1.20	36.9	54.9 ± 10.1	73.1
IAA	0.89 ± 0.28	7.0	1.14 ± 0.06	1.5

Yeast was cultivated in S medium at 30°C for 3 h. 2-H liberation was determined by the flux assay using L-[methylene- ^{14}C] and L-[side chain-2,3- ^3H]tryptophan. Metabolites were analyzed by HPLC directly or after ethyl acetate extraction. Data are means $\pm$ S.D. for three experiments.

Fig. 3. Dose Response of Side Chain 2-H Liberation and Kynureninase Flux to Tryptophan Concentration in Reaction Medium

Yeasts were cultivated in S medium with various concentrations of tryptophan at 30°C for 1 h in a test tube containing 300 μl of broth. $\text{---}\circ\text{---}$, 2-H liberation (transaminase flux), $\text{---}\bullet\text{---}$, alanine liberation (kynureninase flux).

TABLE II. The Metabolism of IPA, IAAld, TOH and IAA

Substrate	Final conc. ($\times 10^{-6}$ M)	Presence of cells	Metabolites ($\times 10^{-6}$ M)		
			TOH	IAA	IAld
IPA	5	—	n.d.	n.d.	3.2
	100	—	n.d.	n.d.	14.6
	500	—	n.d.	n.d.	79.7
	5	+	n.d.	1.0	1.3
	100	+	n.d.	8.2	16.0
IAAld	500	+	n.d.	17.3	25.6
	500	—	n.d.	1.1	0.7
	10	+	1.9	n.d.	n.d.
	100	+	36.6	n.d.	n.d.
	500	+	218	n.d.	n.d.
TOH	100	—	102	n.d.	n.d.
	100	+	102	n.d.	n.d.
IAA	100	—	n.d.	108	n.d.
	100	+	n.d.	47	n.d.

Yeast was incubated with each substrate in 30 ml flasks containing 6 ml of S medium at 30 °C for 3 h. Metabolites were extracted with acidic ethyl acetate and analyzed by HPLC. n.d.; not detected.

late logarithmic phase. Liberation of $2\text{-}^3\text{H}$ was maximally activated in the late logarithmic phase.

The main metabolite formed *via* the 2-H liberation reaction, which had an identical retention time as that of TOH, was further investigated by ^1H -NMR and identified as TOH. Chemical shifts (δ , CDCl_3) were as follows: 1.56 (1H?, brs, OH; overlapped to HDO signal), 3.90–3.93 (2H, t, $J=6.3$ Hz, $-\text{CH}_2\text{-OH}$), 3.03–3.06 (2H, t, $J=6.3$ Hz, Ar-CH_2-), 7.09–7.64 (5H, aromatic protons), 8.05 (1H, brs, NH).

The kynureninase flux is the pathway leading to the synthesis of nicotinamide adenine dinucleotide (NAD), an important cofactor for oxidoreductases, and the main metabolite of 2-H liberation reaction is TOH known as an autoregulatory substance in *Candida albicans*.¹⁹ Differences in the activities of the two fluxes during cell growth may be due to the biological significance of the metabolite of each flux.

In the experiment with 5×10^{-5} M tryptophan, 47.9% of metabolized tryptophan went through the $2\text{-}^3\text{H}$ liberation reaction (Table I) and the sum of KA, TOH and IAA was close to this figure. With 5×10^{-4} M tryptophan, metabolism *via* the $2\text{-}^3\text{H}$ liberation reaction increased drastically and the percentage of TOH to total metabolites was also increased.

Dose response of the alanine liberation and the $2\text{-}^3\text{H}$ liberation is shown in Fig. 3. $2\text{-}^3\text{H}$ liberation from tryptophan occurred dose-dependently at 10^{-5} M and over. The kynureninase flux also increased but reached a plateau at about 10^{-5} M tryptophan. TOH was produced in detectable amounts at higher than 4×10^{-5} M tryptophan and increased dose-dependently (data not shown). On incubation with 5×10^{-3} M tryptophan for 3 h, 360 nmol/ml of TOH was synthesized, most of which distributed extracellularly. Under the same condition, 0.9 nmol/ml of KA was formed. The data indicate that tryptophan metabolism *via* the alanine liberation flux is strictly regulated, while that *via* 2-H liberation reaction was not saturated even with 10^{-3} M tryptophan.

A qualitative study was made to determine the capacity of the yeast to metabolize TOH, IAA, IAAld²⁰ and IPA (Table II). IPA was converted into IAlD spontaneously or to IAA in the presence of cells. TOH, however, could not be detected even when IPA concentration was raised to 5×10^{-4} M. On the other hand, IAAld was readily converted into TOH without forming IAA. No metabolites were detected from either TOH or IAA. However, IAA was reduced to half in 3 h incubation with cells, which indicates that IAA may be metabolized further to another metabolite²¹ not yet identified in the yeast. Consequently, tryptophan is mainly metabolized to TOH and a small amount of IAA was produced *via* IPA and IAAld with IAlD as by-product. Evaluations of both metabolic fluxes of tryptophan and metabolite assay, showed that the kynureninase flux was much smaller than other fluxes under several conditions in this study. However, as the total amount of tryptophan metabolized *via* the fluxes (incorporation into protein, alanine liberation and $2\text{-}^3\text{H}$ liberation) was less than the amount of tryptophan consumed from the medium, metabolic intermediates before alanine liberation (KN or OHKN) were measured. KN formed was 2.3×10^{-7} or 1.03×10^{-6} M, respectively, with 5×10^{-5} or 5×10^{-4} M tryptophan for 3 h, 20–30% of which was distributed intracellularly. The quantity of OHKN formed was reported to be much less than that of KN.²² Taking this into consideration, the tryptophan–NAD flux seems very small. The flux has a significant role in yeast,¹⁰ so that it must be under the regulation of certain factors. This possibility is now under investigation.

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