A NEW PATHWAY OF D-TRYPTOPHAN METABOLISM: ENZYMIC FORMATION OF KYNURENIC ACID VIA D-KYNURENINE

Minoru Tashiro, Kinji Tsukada, Shuhei Kobayashi and Osamu Hayaishi

Department of Medical Chemistry, Faculty of Medicine
Kyoto University, Kyoto, Japan

Received October 9, 1961

In the course of our study on kynurenic acid metabolism we observed that very concentrated sonic extracts of a pseudomonad, employing the so-called "quinoline pathway", were able to degrade D-tryptophan. In this communication we wish to describe a new enzyme system which catalyzes the conversion of D-tryptophan to D-kynurenine, together with a new enzyme, D-kynurenine oxidase, which catalyzes the oxidative deamination of D-kynurenine to kynurenic acid. Recently Behrman and Cullen (1961) found tryptophan racemase in another strain of pseudomonad, which metabolizes D-tryptophan via the so-called "aromatic pathway", but in our strain of pseudomonad D-tryptophan is metabolized without racemization to the L-isomer.

Materials and methods. Cells of Pseudomonas fluorescens (ATCC 11299B) were grown in a medium containing 0.1% D-tryptophan. Other culture conditions and the method of preparing cell-free extracts were the same as reported previously (Hayaishi et al., 1961), except that the cell paste was suspended in an equal volume of buffer before sonication in order to obtain a more concentrated sonic extract.

D-Tryptophan-1'-C^{14} was prepared from DL-tryptophan-1'-C^{14} by the enzymatic conversion of the L-isomer to kynurenic acid, according to the method described previously for the preparation of

kynurenic acid-C^{14} (Hayaishi et al., 1961). D-Tryptophan-1'-C^{14} thus obtained was isolated by ion exchange chromatography on Dowex 1. In order to remove traces of unreacted L-tryptophan-C^{14}, it was further incubated with a resting cell suspension of Pseudomonas fluorescens (ATCC 11250) adapted to DL-tryptophan. D-Tryptophan, which remained in the incubation mixture, was purified by ion exchange chromatography on Dowex 1 and Dowex 50. No other radioactive impurities could be detected by paper chromatography using the three solvent systems mentioned below.

D-Kynurenine was prepared by incubating DL-kynurenine with a resting cell suspension of Pseudomonas (ATCC 11250) adapted to DL-tryptophan. The L-isomer was completely destroyed by this procedure and the remaining D-isomer was isolated by ion exchange chromatography on a Dowex 50 column. No contamination of the product with L-kynurenine could be detected by paper chromatography* and by enzymatic assay with kynureninase.

The standard assay system consisted of 5 μmoles of substrate, 50 μmoles of potassium phosphate buffer, pH 7.5, 0.5 ml of sonic extract (15 to 25 mg protein) in a total volume of 1.0 ml. Incubations were carried out in 10 ml-conical flasks at 37°C with reciprocal shaking, with the exception of anaerobic experiments which were carried out in evacuated Thunberg tubes. At various time intervals aliquots were withdrawn, deproteinized with trichloroacetic acid (TCA) and assayed for reaction products. The identification of the reaction products was made by paper chromatography on Watman No. 1 filter paper. Solvent systems used were butanol saturated with 0.1 N HCl (Knox and Mehler, 1950) and methanol: n-butanol: benzene: water (2:1:1:1) (Mason and Berg, 1951), containing 1%

---

* Separation of D- and L-isomers of kynurenine was achieved with the solvent systems of Price and Dodge (1956) mentioned below.

acetic acid or ammonium hydroxide (Price and Dodge, 1956). The absorption spectra of the aliquots were taken from 260 mμ to 400 mμ with a recording spectrophotometer. Kynurenic acid and anthranilic acid were determined quantitatively on the basis of molar extinction coefficients at 330 mμ and at 310 mμ, respectively.

Formation of kynurenic acid from D-tryptophan. The following experiment was carried out in order to identify the reaction product(s) formed from D-tryptophan by sonic extracts of D-tryptophan adapted cells. D-Tryptophan-1'-C^{14} (10.03 μmoles, specific activity, 10,200 cpm/μmole) was incubated for 3 hours under the conditions described in "Methods". After deproteinization with TCA and neutralization, the reaction mixture was applied to a Dowex 1 formate column (1 x 6 cm). Gradient elution was carried out with 200 ml of water in the mixing flask and 12 N formic acid in the reservoir. Unreacted D-tryptophan was recovered in the first 50 ml of the eluate (45,500 cpm). A single, sharp peak of radioactivity containing 36,400 cpm was eluted between 160 to 265 ml. This radioactive peak coincided exactly with the elution pattern of absorption at 310 mμ, the absorption maximum of kynurenic acid in acid solution. The ratio of radioactivity to absorbancy at 310 mμ was almost constant for each fraction of the peak. The radioactive fractions were pooled, reduced to a small volume under vacuum and subjected to paper chromatography. Radioactivity was located exclusively in a single fluorescent spot having the same Rf value as kynurenic acid in the three solvent systems employed. The specific activity of the enzymatically produced kynurenic acid was 10,000 cpm/μmole which is almost the same as the specific activity of the substrate D-tryptophan-1'-C^{14}, 10,200 cpm/μmole.

Evidences that D-kynurenine is an intermediate. As shown in Table I, D-kynurenine was also converted to kynurenic acid by sonic extracts prepared from D-tryptophan adapted cells. This fact

suggests that D-tryptophan may be metabolized to kynurenic acid through D-formylkynurenine and D-kynurenine by a pathway which is analogous to the reaction sequence by which the L-isomer is degraded. However, the possibility that D-tryptophan is racemized to L-tryptophan was not completely excluded.

In an effort to obtain direct evidence that D-kynurenine is an intermediate, D-tryptophan-1'-C^{14} was incubated with sonic extracts from D-tryptophan adapted cells in the presence of varying amounts of unlabeled DL-kynurenine. But due to the strong inhibition of the D-tryptophan degradation by kynurenine, only a small amount of radioactivity was trapped in kynurenine and almost all radioactivity was recovered as unreacted D-tryptophan.

However, the findings presented in Table I strongly suggest that D-kynurenine rather than L-kynurenine is the intermediate involved. It is well established that the formation of kynurenic acid from L-kynurenine is catalyzed by an α-ketoglutarate dependent transaminase (Miller et al., 1953). In fact, when L-tryptophan or L-kynurenine was incubated in the presence of an α-ketoglutarate-eliminating system, formation of kynurenic acid was never observed and the only product formed was anthranilic acid, which may be produced by the action of L-kynureninase present in the extracts. On the contrary, D-tryptophan or D-kynurenine was converted to kynurenic acid even in the presence of the α-ketoglutarate-eliminating system and neither accumulation of kynurenine nor formation of anthranilic acid was observed (Table I).

D-Kynurenine oxidase. The finding that D-kynurenine is converted to kynurenic acid in the presence of the α-ketoglutarate-eliminating system, while L-kynurenine is not, suggests that a different mechanism must be involved in the formation of kynurenic acid from the D-isomer. Moreover, when D-kynurenine was incubated anaerobically with sonic extracts from D-tryptophan adapted cells, formation of

Table I

Formation of Kynurenic Acid from D-Tryptophan and D-Kynurenine in the Presence of α-Ketoglutarate-Eliminating System.

Incubations were carried out for 2 hours using the standard assay system, containing 5 μmoles of each substrate, with the addition of an α-ketoglutarate-eliminating system which consisted of 500 units (1 mg protein) of glucose dehydrogenase (Strecker, 1955a), 480 units (1.3 mg protein) of glutamic dehydrogenase (Strecker, 1955b), 0.5 μmole of DPN, 100 μmoles of glucose, 50 μmoles of ammonium sulfate.

Substrate	Reaction Products (μmoles)	
	Kynurenic Acid	Anthranilic Acid
D-Tryptophan	1.7	0
L-Tryptophan	0	4.2
D-Kynurenine	4.3	0
L-Kynurenine	0	4.5

kynurenic acid was never observed even in the presence of an excess of α-ketoglutarate, while L-kynurenine was completely converted to kynurenic acid under these conditions. Furthermore, in a preliminary experiment, bleaching of dyes such as methylene blue or 2,6-dichlorophenolindophenol was observed when D-kynurenine was incubated anaerobically with an acetone powder extract from D-tryptophan adapted cells. From these data it was concluded that the formation of kynurenic acid from D-kynurenine is catalyzed by an oxidase. This oxidase is presumed to be specific for D-kynurenine since the crude extract failed to catalyze oxygen consumption with the following amino acids as substrates: D-alanine, D-leucine and D-aspartic acid.

In summary, these data indicate that D-tryptophan is metabolized in this pseudomonad by the following pathway: D-tryptophan → D-formylkynurenine → D-kynurenine → kynurenic acid.

The purification of the various enzymes involved and the elucidation of the mechanisms of these reactions are now under way.

This investigation has been supported in part by research grants from the National Institutes of Health (C-4222), the Rockefeller Foundation, the Jane Coffin Childs Memorial Fund for Medical Research and the Scientific Research Fund of Ministry of Education of Japan.

References

Behrman, E. J. and Cullen, A. M., Federation Proc., 20, 6 (1961).
Hayaishi, O., Taniuchi, H., Tashiro, M. and Kuno, S., J. Biol. Chem., 236, in press (1961).
Knox, W. E. and Mehler, A. H., J. Biol. Chem., 187, 419 (1950).
Mason, M. and Berg, C. P., J. Biol. Chem., 188, 783 (1951).
Miller, I. L., Tsuchida, M. and Adelberg, E. A., J. Biol. Chem., 203, 205 (1953).
Price, J. M. and Dodge, L. W., J. Biol. Chem., 223, 699 (1956).
Strecker, H. J., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. I, Academic Press Inc., New York, 1955a, p. 335.
Strecker, H. J., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. I, Academic Press Inc., New York, 1955b, p. 220.