Biochem. J. (1985) **230**, 635–638
Printed in Great Britain

Human indolylamine 2,3-dioxygenase

Its tissue distribution, and characterization of the placental enzyme

Fumio YAMAZAKI, Takekiyo KUROIWA, Osamu TAKIKAWA and Ryo KIDO
Department of Biochemistry, Wakayama Medical College, Wakayama 640, Japan

(*Received 11 February 1985/18 April 1985; accepted 28 May 1985*)

The presence of indolylamine 2,3-dioxygenase was examined in human subjects by determining its activity with L-tryptophan as substrate. Enzyme activity was detected in various tissues, and was relatively high in the lung, small intestine and placenta. Human indolylamine 2,3-dioxygenase, partially purified from the placenta, had an M_r of about 40000 by gel filtration and exhibited a single pI of 6.9. The human enzyme required a reducing system, ascorbic acid and Methylene Blue, for maximal activity and was able to oxidize D-tryptophan, 5-hydroxy-L-tryptophan as well as L-tryptophan, but kinetic studies indicated that the best substrate of the enzyme was L-tryptophan.

Indolylamine 2,3-dioxygenase activity is distributed in various tissues of mammals such as rabbits (Hayaishi *et al.*, 1975), rats (Cook *et al.*, 1980) and mice (Yoshida *et al.*, 1980). The enzyme has been purified to homogeneity from the rabbit intestine and well characterized; it is a haem-containing protein and catalyses essentially the same dioxygenation reaction as classical hepatic L-tryptophan 2,3-dioxygenase [pyrrolase, L-tryptophan:O_2 2,3-oxidoreductase (decyclizing), EC 1.13.11.11], but differs from the latter with respect to molecular size, substrate specificity, cofactor requirement (Shimizu *et al.*, 1978) and immunogenicity (Watanabe *et al.*, 1981).

Although the precise physiological function of indolylamine 2,3-dioxygenase is still unknown, the dioxygenase of various tissues of mice is induced under some pathological conditions, e.g. viral infection and endotoxic shock (Yoshida & Hayaishi, 1978; Yoshida *et al.*, 1979). On the other hand, Takikawa *et al.* (1984) claim that for mice the physiological substrate *in vivo* may be L-tryptophan rather than its other postulated derivatives such as 5-hydroxy-L-tryptophan and 5-hydroxytryptamine (serotonin) (Hirata & Hayaishi, 1972; Fujiwara *et al.*, 1979).

The present study was undertaken to determine whether indolylamine 2,3-dioxygenase exists in human beings. We report here the presence of the enzyme in various human tissues, and describe some properties of the enzyme from the placenta.

Experimental

Materials

D- and L-Tryptophan, 5-hydroxy-D-tryptophan and tryptamine hydrochloride (Nakarai Chemicals, Kyoto, Japan), L-ascorbic acid and Methylene Blue (Wako Chemicals, Osaka, Japan), kynurenamine dihydrobromide, 5-hydroxytryptamine hydrochloride and 5-hydroxy-L-tryptophan (Sigma Chemical Co.), bovine catalase and calibration protein II (Boehringer Mannheim), bovine serum albumin (4 × crystallized) (ICN Pharmaceuticals), phospho-cellulose (p11) (Whatman), Sephadex G-100, Sephadex G-150 and Pharmalyte (pH 5–8 and pH 7–7.7) (Pharmacia), hydroxyapatite (Seikagaku Kogyo, Tokyo, Japan), Sep-Pak C_{18} (Waters) and Centriflow CF-25 (Amicon) were purchased from the indicated manufacturers. L-Kynurenine was synthesized from L-tryptophan by ozonolysis (Warnell & Berg, 1954). 5-Hydroxy-L-kynurenine and 5-hydroxykynurenamine were generously given by Professor O. Hayaishi (Osaka Medical College, Osaka, Japan).

Human tissues

Human tissues, which were judged normal by eye, were excised within 10 h after death from the corpses autopsied at the Department of Pathology and Department of Forensic Medicine of our college. The intestines were washed with cold 0.9% NaCl to remove the contents. All the tissues were stored at −80°C until used. The placenta, umbili-

cal cord and foetal membranes were obtained from the obstetric clinic and stored at −40°C until used.

Assay of indolylamine 2,3-dioxygenase activity

Human tissues (0.3–0.7g) were homogenized in an ice bath with 2ml of 20mM-potassium phosphate buffer, pH7.0, containing 0.14M-KCl for 45s with a Kinematica Polytron homogenizer with a PT 10 shaft. The tissue homogenates (0.2ml) were added to the standard reaction mixture (total 1ml) containing 50mM-potassium phosphate buffer, pH6.5, 25μM-Methylene Blue, 20mM-ascorbic acid, 50μg of catalase and 0.4mM-L-tryptophan. The reaction, at 37°C, was started with addition of L-tryptophan and terminated after 30min by adding 2ml of 10% (w/v) trichloroacetic acid, and further incubated at 50°C for 30min to hydrolyse *N*-formylkynurenine to kynurenine. After centrifugation at 2500*g* for 15min, kynurenine in the supernatant (2ml) was adsorbed on a Sep-Pak C_{18} column, which was washed with 1.5ml of water and then eluted with 2.5ml of 40% (v/v) ethanol. The eluate was evaporated with a centrifugal freeze-dryer, and redissolved in 0.2ml of mobile phase of h.p.l.c. A portion (50μl) of the solution was analysed by using a Jasco h.p.l.c. system. Kynurenine was separated on a reverse-phase column of ODS-120T (4.6mm × 25cm; Toyo Soda, Tokyo, Japan) with a mobile phase containing 10% (v/v) methanol and 10mM-ammonium acetate, and detected by its absorbance at 360nm. The standard curve was prepared by adding known amounts (0.2 to 10nmol) of kynurenine to the standard reaction mixture. All determinations were performed in duplicate. The data presented are average values.

Purification of human indolylamine 2,3-dioxygenase from the placenta

All the procedures were carried out at 0–5°C. Potassium phosphate buffer, pH6.5, containing 0.2mM-EDTA was used throughout. The placenta (260g) was cut in pieces and rinsed with 0.9% NaCl to remove the blood. The tissues were homogenized with 3vol. of 0.25M-sucrose for 3min in a Waring blender. After centrifugation at 10000*g* for 30min, the supernatant (685ml) was combined with 28.5ml of 0.5M buffer and mixed with 190g of phospho-cellulose previously equilibrated with 20mM buffer. After a gentle stirring for 15min, the suspension was poured into a column (5cm × 20cm). The packed column was washed with 500ml of 20mM-buffer and the enzyme was eluted with 50mM-buffer. The eluate was then applied on a column (2cm × 6cm) of hydroxyapatite previously equilibrated with 50mM buffer. The column was washed with 20ml of the same buffer, and eluted with 150mM buffer. The active fractions were concentrated with a small column of hydroxyapatite and subjected to isoelectric focusing (pH5–8) as described by Noguchi *et al.* (1976). After 48h electrophoresis, the active fractions were concentrated with a Centriflow CF-25, and applied on a column (2.5cm × 90cm) of Sephadex G-100. The gel filtration was performed with 50mM buffer. The active fractions were pooled and concentrated with a Centriflow CF-25. The overall purification was 757-fold from the crude extracts with a yield of 9%. The purified enzyme preparation could be stored at −80°C for at least 4 weeks without loss of activity. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Analytical gel filtration on a Sephadex G-150 column (2.5cm × 100cm) was performed with 50mM-potassium phosphate buffer, pH6.5, as described by Andrews (1964).

Results and discussion

The indolylamine 2,3-dioxygenase activity was determined with various tissues of nine human subjects, with L-tryptophan as substrate. The enzyme activity was detected with various tissues of all human subjects (Table 1). Although there was a considerable individual variation in the activity of each tissue, the activity was relatively high in the lung and small intestine, moderate in the spleen and stomach, and low in the other tissues. For case 9, we were able to examine the central nervous tissues, but could not detect any activity. We also investigated the placenta and its related tissues. The activity in the placenta was relatively high [260.7 ± 56.6 (mean ± S.E.M., $n = 4$) nmol of kynurenine/h per g of tissue], but that in the umbilical cord and foetal membranes was low (< 10nmol of kynurenine/h per g of tissue).

To characterize human indolylamine 2,3-dioxygenase, we attempted to purify the enzyme from the placenta. By four purification steps, i.e. phospho-cellulose chromatography, hydroxyapatite chromatography, isoelectric focusing and Sephadex G-100 gel filtration, a 757-fold-purified enzyme preparation was obtained from the crude extracts (Table 2). The M_r of the purified enzyme was determined to be about 40000 by analytical gel filtration with Sephadex G-150. This size was close to that of the rabbit enzyme, which had an M_r of 41000 ± 1000 (Shimizu *et al.*, 1978). When the human enzyme was subjected to isoelectric focusing (pH6.7–7.7), the activity was focused sharply at pH6.9. This result was in contrast with the report that the rabbit enzyme had an acidic pI and exhibited a heterogeneity on isoelectric focusing; the enzyme activity was separated into three

Table 1. *Tissue distribution of indolylamine 2,3-dioxygenase activity in human subjects*
Activities were determined as described in the Experimental section. The death cause, age and sex of each subject were: case 1, heart anomaly (2 months old, male); case 2, trauma (18 years old, male); case 3, trauma (43 years old, male); case 4, lung cancer (44 years old, male); case 5, brain haemorrhage (53 years old, male); case 6, lung cancer (65 years old, male); case 7, hepatoma (68 years old, male); case 8, myocardial infarction (78 years old, male); case 9, hepatoma (73 years old, female). —, Not determined; N.D., not detected.

	Enzyme activity (nmol of kynurenine/h per g of tissue)								
Case no. ...	1	2	3	4	5	6	7	8	9
Lung	—	93.5	92.7	22.2	185.4	—	90.4	16.2	1229.0
Heart	—	1.5	3.3	N.D.	1.6	—	N.D.	—	N.D.
Aorta	—	1.3	7.2	N.D.	N.D.	—	—	9.7	N.D.
Spleen	19.0	54.8	9.5	27.7	11.0	17.7	31.2	13.1	N.D.
Pancreas	20.0	1.2	1.9	2.0	N.D.	15.0	N.D.	—	—
Kidney									
Cortex	83.5	—	—	N.D.	52.9	N.D.	N.D.	N.D.	—
Medulla	64.2	1.0	2.6	N.D.	45.0	N.D.	N.D.	1.9	—
Adrenal	N.D.	1.7	6.8	8.8	N.D.	—	2.6	—	—
Urinary bladder	—	—	—	4.2	N.D.	—	N.D.	—	—
Prostate	—	—	—	4.5	1.5	—	N.D.	—	—
Testis	—	0.3	—	—	N.D.	N.D.	—	—	—
Epididymis	N.D.	—	—	3.6	9.0	—	—	—	—
Stomach									
Corpus	—	—	—	—	17.6	24.5	—	14.6	4.3
Pyloric part	6.5	—	4.6	—	—	10.2	N.D.	11.1	32.5
Duodenum									
Upper	48.5	—	—	33.3	—	38.1	7.6	—	46.1
Lower	—	—	—	62.5	—	37.3	—	—	60.5
Jejunum									
Upper	87.5	—	—	64.5	30.7	47.8	19.0	6.8	26.3
Lower	32.6	—	—	47.8	34.6	16.0	3.3	24.3	21.0
Ileum									
Upper	11.5	—	—	40.0	—	26.7	N.D.	8.3	13.5
Lower	11.7	—	—	30.6	—	3.9	3.3	20.1	73.0
Appendix	6.2	—	—	12.7	21.4	9.7	N.D.	—	—
Caecum	—	—	—	13.1	—	1.6	—	6.1	46.1
Colon									
Ascending	N.D.	—	—	5.9	40.9	3.0	N.D.	7.5	6.0
Transverse	5.5	—	—	10.2	—	N.D.	N.D.	8.1	N.D.
Descending	—	—	—	6.2	—	N.D.	N.D.	N.D.	N.D.
Sigmoid	7.4	—	—	8.7	—	N.D.	N.D.	N.D.	N.D.
Rectum	5.2	—	—	N.D.	—	1.2	—	2.6	N.D.

Table 2. *Purification of indolylamine 2,3-dioxygenase from human placenta*
The details are described in the Experimental section.

Step	Volume (ml)	Total protein (mg)	Specific activity (nmol of kynurenine/min per mg of protein)	Yield (%)	Purification (fold)
Crude extracts	685	5891	0.35	100	1
Phospho-cellulose	262	157	8.57	65	24
Hydroxyapatite	230	32	38.5	60	110
Isoelectric focusing	13.8	3.7	172.1	31	492
Sephadex G-100	0.9	0.7	264.8	9	757

fractions, with pI 4.9, 5.1 and 5.3 (Shimizu *et al.*, 1978).

Kinetic analyses (Taniguchi *et al.*, 1979; Sono *et al.*, 1980) have revealed that the dioxygenase from the rabbit intestine has protohaem IX as a catalytic centre, that the ferrous enzyme is the active form for dioxygenation, and that, because of its strong autoxidizability to the ferric form, which has no

dioxygenase activity, the enzyme requires a reducing system, e.g. ascorbic acid and Methylene Blue, to maintain the 'active' ferrous form during catalysis. The role of Methylene Blue is considered to be an electron carrier from ascorbic acid to the ferric enzyme. Similarly, the purified human enzyme required both ascorbic acid and Methylene Blue for the dioxygenation reaction. The concentrations of ascorbic acid and Methylene Blue for maximal activity were 10mM and 5μM respectively. These concentrations were nearly equal to those found necessary for the rabbit enzyme (Yamamoto & Hayaishi, 1967). It was also reported for the rabbit enzyme that, when the reducing system is used, catalase is required to protect the enzyme from an inactivation caused by H_2O_2, which is generated by the reducing system as a result of reduction of O_2 (Hirata *et al.*, 1977). This sensitivity to H_2O_2 of the rabbit enzyme appears to be true of the human enzyme also, because the enzyme activity decreased by about 50% in the absence of catalase. The concentration of catalase for maximal activity was 10μg/ml.

As seen for the rabbit enzyme (Shimizu *et al.*, 1978), the human enzyme exhibited a broad substrate specificity; it was able to oxidize D-tryptophan and 5-hydroxy-L-tryptophan as well as L-tryptophan. The apparent maximal activities ($V_{max.}$) of D-tryptophan, L-tryptophan and 5-hydroxy-L-tryptophan were 1140, 392 and 24nmol of product/min per mg of protein respectively, which were determined by Lineweaver–Burk plots. The highest activity was obtained with D-tryptophan, but its affinity for the enzyme was very low compared with that of the L-isomer; the apparent K_m for D-tryptophan was 14.3mM, which was 550 times that (26μM) of the L-isomer. The optimal pH values for D- and L-tryptophan were around 7.5 and 6.5 respectively with 50mM-potassium phosphate buffer. Similar stereospecificity for tryptophan was observed with the rabbit enzyme (Shimizu *et al.*, 1978). For 5-hydroxy-L-tryptophan, its $V_{max.}$ value and K_m value (400μM) indicated that the hydroxylation at 5-position of L-tryptophan decreased its maximum activity and lowered its affinity for the enzyme. The optimal pH was around 5.5 with 50mM-potassium phosphate buffer. In addition, 5-hydroxy-D-tryptophan, 5-hydroxytryptamine and tryptamine also served as dioxygenation substrates for the human enzyme. However, their rates were even lower than that of 5-hydroxy-L-tryptophan, and, because of the limited amount of the purified enzyme, their kinetic parameters could not be determined in the present study.

In summary, the presence of indolylamine 2,3-dioxygenase was demonstrated in various human tissues. The human enzyme was essentially the same as the rabbit enzyme with respect to catalytic properties, and kinetic studies indicated that its best substrate was L-tryptophan.

References

Andrews, P. (1964) *Biochem. J.* **96**, 596–606

Cook, J. S., Pogson, C. I. & Smith, S. A. (1980) *Biochem. J.* **189**, 461–466

Fujiwara, M., Shibata, M., Nomiyana, Y., Sugimoto, T., Hirata, F., Tokuyama, T., Senoh, S. & Hayaishi, O. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1145–1149

Hayaishi, O., Hirata, F., Fujiwara, M., Ohnishi, T. & Nukiwa, T. (1975) *Proc. FEBS Meet.* **10**, 131–144

Hirata, F. & Hayaishi, O. (1972) *Biochem. Biophy. Res. Commun.* **47**, 1112–1119

Hirata, F., Ohnishi, T. & Hayaishi, O. (1977) *J. Biol. Chem.* **253**, 4637–4642

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Noguchi, J., Okuno, E., Minatogawa, Y. & Kido, R. (1976) *Biochem. J.* **155**, 107–115

Shimizu, T., Nomiyama, S., Hirata, F. & Hayaishi, O. (1978) *J. Biol. Chem.* **253**, 4700–4706

Sono, M., Taniguchi, T., Watanabe, Y. & Hayaishi, O. (1980) *J. Biol. Chem.* **255**, 1339–1345

Takikawa, O., Yoshida, R., Yasui, H. & Hayaishi, O. (1984) in *Progress in Tryptophan and Serotonin Research* (Schlossberger, G. H., Kochen, W., Linzen, B. & Steinhart, H., eds.), pp. 517–520, Walter de Gruyter, Berlin and New York

Taniguchi, T., Sono, M., Hirata, F., Hayaishi, O., Tamura, M., Hayashi, K., Iizuka, T. & Ishimura, Y. (1979) *J. Biol. Chem.* **254**, 1339–1345

Warnell, J. L. & Berg, C. P. (1954) *J. Am. Chem. Soc.* **76**, 1708–1709

Watanabe, Y., Yoshida, R., Sono, M. & Hayaishi, O. (1981) *J. Histochem. Cytochem.* **29**, 623–632

Yamamoto, S. & Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 5260–5266

Yoshida, R. & Hayaishi, O. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3998–4000

Yoshida, R., Urade, Y. Tokuda, M. & Hayaishi, O. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4084–4086

Yoshida, R., Nukiwa, T., Watanabe, Y., Fujiwara, M., Hirata, F. & Hayaishi, O. (1980) *Arch. Biochem. Biophys.* **203**, 343–353