Brain tryptophan hydroxylase: Purification of, production of antibodies to, and cellular ultrastructural localization in serotonergic neurons of rat midbrain

(immunocytochemistry/microtubules/raphe nucleus/serotonin/tryptophan 5-monooxygenase)

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ABSTRACT Tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating)], the enzyme catalyzing the rate-limiting step in the biosynthesis of serotonin, was purified 79-fold from the region of the raphe nucleus of rat midbrain by sequential column chromatography and disc-gel electrophoresis. In electrophoresis three bands were distinguished, A, B, and C, which, when separated and submitted individually to electrophoresis, reproduced the same three bands. Bands A and C were enzymatically active and inhibited by para-chlorophenylalanine. Antibodies produced to each of the three bands crossreacted by immuno double diffusion and electrophoresis with each other and homogenates of raphe nuclei; they completely inhibited enzyme activity only of tryptophan hydroxylase. Tryptophan hydroxylase was localized by light and electron immunohistochemistry to serotonin neurons of the raphe. Ultrastructurally, in cell bodies, the enzyme was distributed in cytoplasm and in association with endoplasmic reticulum and Golgi apparatus. In dendrites and axons, it was associated with microtubules. Tryptophan hydroxylase in brain is only neuronal and cytoplasmic, exists in multiple forms, and is associated with microtubules, suggesting it may be transported from sites of synthesis in cell body into axons.

The enzyme, tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating)] catalyzes the initial and probably rate-limiting step in the biosynthesis of the neurotransmitter serotonin (5HT) (1, 2). In brain, the enzyme has been presumed to be contained only within those specific neurons which synthesize, store, and release 5HT (3). The evidence is indirect and based on the reasonable assumption that those neurons in which 5HT can be visualized by their specific histofluorescence (4, 5) should contain the enzyme. Consistent with the surmise are biochemical data demonstrating the presence of high levels of tryptophan hydroxylase activity in brain regions containing the cell bodies of 5HT neurons (3) or richly innervated by their processes (3). It has also been proposed, on indirect evidence, that tryptophan hydroxylase exists in different forms, differing either in susceptibility to inhibition by drugs (6–8) or possibly, in their relationship to subcellular organelles (3).

Direct information of the biochemical structure and regional and subcellular localization of the enzyme has been restricted by limited success (9, 10) in obtaining purified tryptophan hydroxylase suitable for the development of specific antibodies for immunohistochemistry. Recently the development in our laboratory of techniques for the purification and immunohistochemical localization of tyrosine hy-
was ing 2 mm length and placed in 250 µl of 0.3 M Tris-acetate buffer, pH 7.0 containing 2 mM dithiothreitol to elute enzyme. The tubes were allowed to stand overnight at 4°C, and the enzyme activity was determined in the buffer after the gel slices were removed.

Production of Antibodies. Purified tryptophan hydroxylase was subjected to polyacrylamide disc gel electrophoresis. Segments containing active enzyme were pooled from 24 gels and homogenized in a glass tube with 2.0 ml of 0.9% sodium chloride. An equal volume of complete Freund's adjuvant was added; the mixture was thoroughly emulsified and injected subcutaneously into white New Zealand rabbits. The injections were repeated every 3 weeks for a period of 9 weeks. The rabbits were bled 5 days after the final injection. Immunoglobulin (IgG) was precipitated from the serum at 50% saturation with ammonium sulfate. Immunodiffusion and immunoelectrophoresis were carried out by the methods of Ouchterlony (21) and Scheidegger (22), respectively.

Immunohistochemical Localization of Tryptophan Hydroxylase By Light and Electron Microscopy. Tryptophan hydroxylase was localized immunohistochemically in rat brain by use of the peroxidase–antiperoxidase method of Sternberger et al. (23). The techniques were identical to those used by us for localization of tyrosine hydroxylase, and the methods are extensively detailed elsewhere (13, 15).

In summary, rats were anesthetized with pentobarbital (50 mg/kg) and perfused through the aorta with either 4% paraformaldehyde in phosphate buffer, pH 7.3, or with dilute Karnovsky fixative (1% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.15). For light microscopy, the formalin-perfused brain was post-fixed for 6 hr with picric acid/formalin, washed overnight in phosphate buffer, embedded in polyethylene glycol, and sectioned on a rotary microtome at 5 µm. For electron microscopy, perfusion of the brain with Karnovsky's solution was followed by fixation for 2 hr in 2% glutaraldehyde paraformaldehyde. Sections of 20 µm were taken through the midbrain on a vibrating microtome (Vibratome) with the tissue immersed in 0.1 M cacodylate buffer. Sections prepared for light or electron microscopy were sequentially incubated with rabbit antisera to tryptophan hydroxylase, goat antiserum to rabbit IgG, and peroxidase–antiperoxidase complex, and then reacted for 15 min with 3,3'-diaminobenzidine and hydrogen peroxide to yield a brown reaction product. For light microscopy the sections were lightly counterstained with cresyl violet, dehydrated, and mounted in Permount. For electron microscopy, an area of the raphe nucleus was dissected from each section, fixed for 30 min in 2% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were taken from the outer surface (1–2 µm) of each 20-µm section for subsequent examination with a Philips 301 electron microscope without additional staining. Control sections were incubated with either preimmune serum or tryptophan hydroxylase antiserum maximally adsorbed with the purified enzyme (13, 15).

RESULTS

Purification of tryptophan hydroxylase in rat brain

Raphe nuclei from 280 Sprague–Dawley rats were homogenized in 50 mM Tris-acetate buffer, pH 7.6, containing 2 mM dithiothreitol using a Polytron tissue homogenizer.

The same buffer was used for Sepharose and Sephadex column chromatography. The homogenate was centrifuged at 39,000 x g for 30 min, and powdered ammonium sulfate was added to the supernatant to 80% saturation. The enzyme was further purified by three sequential chromatographic steps: In the first step, the enzyme was passed through a Sepharose 4B column achieving 2.8-fold purification of enzyme and 80% recovery of activity. This step removed, in the void volume, an enzymatically inactive peak containing substances of high molecular weight and having a high spectrophotometric reading at 260 nm, indicating the presence of compounds with a relatively high content of nucleic acids. If this fraction was not removed, subsequent purification procedures were ineffective because the enzyme would aggregate into a high-molecular-weight form which was then inseparable from other proteins by chromatography or electrophoresis.

Active fractions were then passed through a hydroxyapatite column by stepwise elution of protein using 0.0625, 0.125, and 0.25 M potassium phosphate buffer, pH 6.8, containing 2 mM dithiothreitol. The highest specific activity was obtained by elution with 0.125 M buffer. The final step consisted of Sephadex G-200 column chromatography in which a 2.4-fold purification was obtained. The specific activity of the purified enzyme was 45 units/mg of protein, which represented a 79.2-fold purification of the enzyme from the 39,000 x g supernatant.

After each chromatographic step the enzyme was subjected to polyacrylamide gel electrophoresis. The number of protein bands detected on the gels after each step was not substantially reduced despite an increase in specific activity. Electrophoresis of the enzyme after the final chromatographic step, the G200 effluent (Fig. 1), produced three heavily stained (major) bands designated A, B, and C (in reverse order of electrophoretic mobility) and several lightly stained (minor) bands. Only two of the major bands, A and C (Fig. 1), exhibited enzyme activity, band C having the greater specific activity. The remaining band, B, was inactive. Enzyme activity of both active bands, A and C, were completely inhibited by p-chlorophenylalanine, an inhibitor of tryptophan hydroxylase (6–8).

Each major band, A, B, and C, was then isolated from 33 gels, eluted, and subjected, by itself, to electrophoresis (Fig. 1). Electrophoresis of individual bands, A, B, or C, resulted in the reappearance of all three bands, although in each case the original band predominated (Fig. 1). This finding suggests that the inactive band B is related in some manner to the active enzyme; the reason for its apparent inactivity may be due to the absence of an active prosthetic group or to insensitivity of our enzyme assay.

Antibodies to tryptophan hydroxylase

We next prepared antibodies to tryptophan hydroxylase using the active enzyme of the C band (Fig. 1) as antigen. The antibody appeared highly specific for tryptophan hydroxylase by the following criteria: (a) Immunoelectrophoresis and double immunodiffusion of antibody run against a crude extract of the raphe nuclei region of rat brain (Fig. 2, H), purified enzyme eluted from the Sephadex G-200 column (Fig. 2, C200), and to each of the specific protein bands A, B, or C (Fig. 2, A, B, and C), detected by gel electrophoresis of the Sephadex eluate (Fig. 1) yielded single precipitin arcs (Fig. 2). On the other hand, no precipitin was formed when the antibody was run against other purified enzymes.
involved in the biosynthesis of monoamines from rat brain, including tyrosine hydroxylase, dopamine-β-hydroxylase, aromatic L-amino-acid decarboxylase, and quinonoid dihydrolase reductase. (b) The antibody completely inhibited the activity of tryptophan hydroxylase either as purified enzyme (bands A and C) or in homogenates of the raphe nuclei region of rat brain, but not the activities of the other aforementioned enzymes. (c) As detailed below, the antibody was specifically localized immunohistochemically only to the cell bodies and processes of neurons defined by others by histofluorescence (4, 5) as containing 5HT.

Antibodies were also prepared against the other active band, A, and the inactive band B. These antibodies cross-reacted with the A, B, and C bands by immunodiffusion and electrophoresis, and inhibited enzyme activity in tissue, and were localized only to 5HT neurons of the midbrain by immunohistochemistry.

Immunohistochemical localization of tryptophan hydroxylase

In sections of midbrain, pons, and medulla reacted with antibody to tryptophan hydroxylase and immunohistochemically stained by the peroxidase-antiperoxidase method, a dark reaction product was observed by light microscopy within specific groups of neurons and their processes. The localization of these neurons corresponded in large measure to those areas in which 5HT neurons have been demonstrated by specific histofluorescence (4, 5), particularly the nuclei of the raphe. A detailed mapping of the location of the cell bodies and projections of processes containing tryptophan hydroxylase will be presented elsewhere, consideration being focused here only on the morphology of typical cells.

In sections through the midbrain raphe area near the medial longitudinal fasciculus, dark reaction product was contained in the cytoplasm of small, highly branched neurons (Fig. 3A). In perikarya, enzyme was localized in a thin rim of cytoplasm surrounding an unstained nucleus and extended into branched dendrites and axons which could be distinguished in the unstained neuropil as black dots in cross-section, or, when cut longitudinally and examined with phase contrast optics, as irregular strands of black material. The stained axons were visualized along their passage through the upper brainstem and forebrain into terminal fields. The cells and processes of stained neurons were often closely associated with heavily myelinated tracts such as the medial longitudinal fasciculus and medial lemniscus, and also the cortico-spinal (pyramidal) pathway. No reaction product was seen in neurons of comparable size and location in sections incubated with control serum and lightly counterstained with cresyl violet (Fig. 3B).

By electron microscopy, examination of neurons in the nucleus raphe dorsalis incubated with antibody to tryptophan hydroxylase demonstrated electron-dense reaction product in the processes and perikarya of stained neurons. At low magnification (Fig. 3C) numerous stained unmyelinated processes were clearly delineated from an unstained neuropil rich in myelinated fibers (Fig. 3C). In the cell soma (Fig. 4A) the peroxidase staining was widely distributed throughout the cytoplasm. Endoplasmic reticulum and Golgi apparatus were also stained, whereas mitochondria, lysosomes, and the plasma membranes were not. Within processes, the enzyme appeared to be associated with specific subcellular organelles. In longitudinal sections through both axons and dendrites (Fig. 3C), dense reaction product was aligned in linear aggregates forming fiber-like structures arranged in parallel with the plasma membrane. In cross-section (Figs. 3C, 4A, and 4B), the reaction product appeared in the form of darkly stained round structures with a mean diameter of 22 nm. In small axons (Fig. 4A) the labeled subcellular organelles appeared to have a more regular geometric array than in dendrites. On the basis of distribution and size, the labeled structures have the characteristics of microtubules (24) and indicate that tryptophan hydroxylase, like tyrosine hydroxylase (15), is in some manner associated with that organelle.

DISCUSSION

In the present study we have been able to isolate the enzyme tryptophan hydroxylase from rat brain of sufficient purity so as to produce antibodies to the enzyme. The principal obsta-
FIG. 3. Immunohistochemical localization of tryptophan hydroxylase to neurons in the region of the raphe nuclei of rat brain mesencephalon stained by the peroxidase-antiperoxidase method. (A) Phase contrast photomicrograph of section incubated with tryptophan hydroxylase antiserum showing darkly stained reaction product in the cytoplasm and processes of neurons (unmarked arrows). Other labeled neuronal processes (P) are seen in close association with unstained myelinated fiber bundles (MB) ×132.5. (B) Control phase contrast photomicrograph of a section through the raphe nuclei incubated with blocked antiserum. Unstained neurons (arrows) are associated with the myelinated fiber bundle (MB) ×132.5. (C) Low magnification electron micrograph of peroxidase staining in neuronal processes of section incubated with tryptophan hydroxylase antiserum. A longitudinal section through one dendritic process shows selective localization along neurotubular structures (arrows). A cross section through a labeled dendrite is indicated by the synapse (S) on its surface. Cross sections of numerous other labeled processes are distributed throughout the section. Unlabeled myelinated axons (MA) are also present ×10,600.

FIG. 4. Ultrastructural localization of tryptophan hydroxylase in soma and processes of neurons in nucleus raphe dorsalis using peroxidase-antiperoxidase staining method. (A) Electron micrograph of section incubated with specific tryptophan hydroxylase antiserum showing staining in cytoplasm of a neuron whose lateral border is indicated by arrows. There is some selective association with endoplasmic reticulum (ER), but not with mitochondria (M). Axon (Ax) in neuropil shows selective staining of rounded subunits 22–24 nm in diameter. ×21,200. (B) Higher magnification photomicrograph of dendrite indicated by the synapse (S) in Fig. 3C. Numerous round peroxidase-stained structures with dimensions of neurotubules (arrows) are present throughout the dendrite indicated by synapse (S). Mitochondria (M) are unstained. ×21,200. (C) Control electron micrograph of a section incubated with blocked antiserum. The dendrite whose surface is marked by the synapse (S) on its surface contains unstained neurotubules (arrows) as well as unstained mitochondria (M). ×21,200. Darkening of the cell membrane in the area of the synaptic cleft occurs in both control and stained sections and hence is not an indication that tryptophan hydroxylase is localized to the synapse.

Slowly moving A band, and the inactive band B, and that each band when isolated and resubjected to electrophoresis resulted in replication of the three bands suggests that tryptophan hydroxylase exists in multiple forms, as others have suggested (3, 8). The nature of the multiple forms of the enzyme, however, is uncertain. The fact that antibodies to one band crossreact with each other and that active bands A and C were inhibited by p-chlorophenylalanine suggests that each form of the enzyme shares a common protein structure, and that at least bands A and C have closely related active sites. However, whether the multiple forms of the enzyme represent different states of aggregation of basic enzyme units into dimers, trimers, etc., as is the case with tyrosine hydroxylase (12, 25), or represent charge isomers, i.e., forms of the enzyme having comparable protein structure but differing in ionic charge, as exemplified by phenylethanolamine-N-methyl transferase (26), remains to be determined.

By immunohistochemistry, tryptophan hydroxylase was
localized by light microscopy to the cytoplasm of small neurons with extensively branched processes, many of which are concentrated within the confines of the nuclei of the raphe. These neurons, on the basis of their configuration and distribution, correspond almost exactly to those defined by histochemistry as containing the neurotransmitter 5HT (4, 5). These findings would support the indirect evidence that in brain, tryptophan hydroxylase is contained exclusively within those neurons that synthesize, store, and release the neurotransmitter 5HT.

Ultrastructurally the distribution of tryptophan hydroxylase is not uniform throughout the cell. Within the cell body the enzyme is diffusely distributed in cytoplasm but tends to be associated with membranes of endoplasmic reticulum and Golgi apparatus, possibly reflecting the sites in which the specific protein is synthesized (27). In processes, both axons and dendrites, the enzyme is primarily associated with subcellular organelles having the characteristics of microtubules (24).

The nature of the association of tryptophan hydroxylase and microtubules in axons and dendrites is obscure. Conceivably it might be an artifact in which the enzyme, which is preponderantly soluble (3, 8), becomes cross-linked to other cellular proteins as a consequence of fixation with glutaraldehyde. However, for cross-linking to occur the enzyme and the cellular protein to which it binds must be in close proximity since free diffusion would be arrested by fixation. Thus the fact that only microtubules are stained for tryptophan hydroxylase while other membranous constituents of the cell, such as lysosomes, mitochondria, or plasma membranes, are not, indicates that the association of enzyme and microtubules has specificity. It is not possible to determine by these methods whether tryptophan hydroxylase is incorporated into microtubules, is adherent to their surface, or is conveyed free in cytosol but in close approximation to the organelle.

The ultrastructural distribution of tryptophan hydroxylase, in particular its localization to microtubules, is strikingly similar to that of the closely related enzyme tyrosine hydroxylase (14, 15), which catalyzes the rate-limiting step in the biosynthesis of catecholamines in the sympathetic nervous system and in brain. The association of both tyrosine and tryptophan hydroxylases with microtubules is of considerable interest with regard to the potential transport of these enzymes from the presumed sites of synthesis in the cell bodies into terminals within the brain. The fact that in peripheral sympathetic neurons tyrosine hydroxylase is transported at relatively rapid rates of flow (27, 28) and that the integrity of microtubules appears important in mediating fast transport (29, 30) has led us to suggest that the association of tyrosine hydroxylase with microtubules in the central nervous system provides indirect evidence for the transport of this enzyme within the brain (14, 15). On the other hand, it is not known whether tryptophan hydroxylase is also transported from the cell body into axons to the periphery of 5HT neurons, because no peripheral model of this neuron exists. However, the present finding of an association of tryptophan hydroxylase with the microtubules in the rat central nervous system raises for the first time the possibility that this enzyme is also transported, conceivably by mechanisms similar to those for tyrosine hydroxylase.

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