A monoclonal antibody to tryptophan hydroxylase: applications and identification of the epitope

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Abstract

Recombinant rabbit tryptophan hydroxylase (TPH) was expressed in Escherichia coli and purified from inclusion bodies by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A mouse monoclonal antibody and rabbit and sheep polyclonal antibodies were generated. In immunohistochemical studies of formaldehyde-fixed primate brain, the monoclonal strongly labeled not only cell bodies in the raphe nuclei but also fibers in the cerebral cortex. Truncation mutants and peptide pre-competition were used to localize the epitope to E 103SVPWFP109. Although the primary sequences of TPH encoded by mRNAs from brain and pineal gland are identical, differences in the immunoreactivity of TPH protein from these two sources were observed in blot immunolabeling studies. TPH immunoreactivity migrated as an Mr ~56 000 band in each of the tissues except human pineal glands, in which the TPH reactivity was ~3 kDa lower. In addition, the relative intensities of TPH immunolabeling across the four tissues differed among these antibodies and a previously described monoclonal antibody against phenylalanine hydroxylase (PH8), which cross-reacts with TPH. Whereas PH8 exhibited roughly equivalent TPH reactivity per protein in both tissues from both species, TPH from human and rat raphe nuclei was preferentially recognized by the present monoclonal. By contrast, the affinity-purified sheep polyclonal antibody reacted preferentially with TPH from human and rat pineal gland, and the affinity-purified rabbit polyclonal antibody appeared to selectively recognize TPH from human pineal gland. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Tryptophan hydroxylase (TPH; EC 1.14.16.4) catalyzes the conversion of L-tryptophan to L-5-hydroxytryptophan, the initial step in serotonin and melatonin biosynthesis (Grahame-Smith, 1964; Hakanson et al., 1967; Jequier et al., 1967, 1969; Lovenberg et al., 1967). In mammals, serotonin biosynthesis occurs predominantly in neurons which originate in the raphe nuclei of the brain, and melatonin synthesis takes place within the pineal gland. Although TPH catalyzes the same reaction within the raphe nuclei and the pineal gland, TPH activity is rate-limiting for serotonin but not melatonin biosynthesis.

A single gene appears to be responsible for TPH expression (Kim et al., 1991) and no differences have been found within the coding regions of the raphe and pineal TPH for three different species (rabbit, Grenett et al., 1987; human, Boulard et al., 1990; rat, Kim et al., 1991). Despite identical coding sequences for pineal gland and raphe TPH within a given species, differences in the biochemical properties of pineal and raphe TPH, including molecular weights, substrate specificities, isoelectric points, and turnover rates (cf. Kim et al., 1991) have been reported. For example, the rate of TPH
turnover is over an order of magnitude higher in the pineal gland (Sitaram and Lees, 1978) than in the raphe (Meek and Neff, 1972). Few of the reported differences, however, have resulted from direct comparisons of pineal and raphe TPH in the same study. Of these, although rat pineal and raphe TPH have highly disparate isoelectric points in chromatofocusing (Kim et al., 1991), both migrate as a 56 kDa band in immunoblots after reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Dumas et al., 1991), both migrate as a 56 kDa band in disparate isoelectric points in chromatofocusing (Kim et al., 1991). The present study has characterized these immunochemical differences, which may reflect variations in the post-translational processing of pineal versus raphe TPH.

2. Materials and methods

2.1. Tissues

Human brain tissue and pineal glands were obtained at autopsy from the Coroner’s Office of Cuyahoga County, OH in accordance with an approved Institutional Review Board protocol and stored at −80 °C. Subjects (age, 42–62 years; postmortem interval, 9–27 h) had suffered sudden deaths from either cardiomyopathy (n = 3) or accident (n = 1) and tested negative in drug toxicology screens. DR samples were subsequently punched from frozen sections as previously described (Stockmeier et al., 1996). Control punches lateral to the DR (containing low levels of TPH) were also taken.

Rat tissue samples were dissected after halothane anesthesia and decapitation. The pineal gland was obtained prior to removal of the brain from the cranium. DR and control (as above) punches were taken after making coronal slices (ASI Instruments; Warren, MI). Samples were immediately frozen and stored at −80 °C.

Monkey tissue samples were obtained from two male cynomolgus monkeys (Macaca fascicularis). They were deeply anesthetized with ketamine hydrochloride (25 mg/kg) and pentobarbital sodium (30 mg/kg), and perfused transcardially with cold 4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4) as previously described (Lewis et al., 1994). The brain was immediately removed and coronal blocks (3–5 mm thick) were excised and immersed in the same fixative for 6 h. Tissue blocks were washed in a series of cold, graded sucrose solutions and then sectioned (40 μm) on a cryostat.

2.2. Recombinant TPH

Expression vectors for full-length rabbit TPH (Vrana et al., 1994) and for amino-terminal deletions (NAl60, NAI106, NAI116; Kumer et al., 1997) were transformed into Escherichia coli (BL21[DE3]; F−, ompT, rB m−). Cells were induced to express recombinant TPH in mid-log growth phase with 1 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h at 30 °C. FeSO4 (100 μM) was added to the culture medium during the induction period. Cells were harvested by centrifugation at 1000 × g and resuspended in 1/100th volume (relative to the original culture volume) of 25 mM HEPES (pH 7.0), 250 mM sucrose, 1 mM DTT, 575 μM PMSF, 10 μM Fe(NH4)2(SO4)2, 2 μg/ml leupeptin, 0.5 μg/ml pepstatin A on ice. Suspensions were brought to 1% Triton X-100 and sonicated. After centrifugation (14000 × g; 4 °C; 15 min), the resulting pellets were extracted (by sonication and centrifugation) in buffer (25 mM Tris–HEPES, 1 mM EDTA, 1 mM benzamidine, 5 μg/ml leupeptin; pH 7.4 at 4 °C) containing 1% Triton X-100 and then twice in buffer containing 3 M urea. The final pellets (inclusion bodies) were solubilized in SDS-PAGE sample buffer. As judged by Coomassie staining of SDS-PAGE gels, recombinant TPH comprised 60–80% of the protein in the inclusion body preparations. Full-length TPH was further purified by preparative SDS-PAGE (BioRad; 8%T) and fractions containing the TPH band (56 kDa) were pooled, concentrated (CentriPrep30; Amicon), and dialyzed extensively against Dulbecco’s phosphate buffer saline (PBS) containing 0.1% SDS.

2.3. TPH antibodies

Polyclonal rabbit and sheep antisera were raised by Bethyl Laboratories (Montgomery, TX) and a monoclonal mouse antibody was generated by Sigma Israel (Rehovot, IS) against the purified (>95%) full-length recombinant TPH. For polyclonal antibodies, animals were immunized initially with 200 μg TPH per animal and boosted with 100 μg after 2, 4, and 6 weeks and 50 μg at 4-week intervals thereafter. Sera were collected every other week for 6–8 months. The development and progression of immune responses were monitored by strip-blot immunolabeling assays of solubilized human DR. For monoclonal antibodies, BALB/c mice were immunized and boosted with 10 μg TPH at 3-week intervals. Fusion between spleen cells of immunized mice and NS-1 mouse myeloma cells was performed 3 days after the third booster injection, using polyethylene glycol (M.W. 1500). Mixed cell cultures were subsequently grown in Dulbecco’s modified eagle medium containing 100 μM hypoxanthine, 16 μM thymidine, 0.4 μM aminopterin and supplemented with 10% heat-inactivated horse serum. After 10–14 days of culture,
supernatants were screened for TPH antibodies by enzyme linked immunosorbent assay (ELISA) and strip-blot immunolabeling assays of TPH immunogen. Positive wells were isolated and cells were cloned by limiting dilution. Supernatants were rescreened for TPH immunoreactivity by strip-blot immunolabeling assays of solubilized human DR, and an IgG3-producing clone (available under the product name WH3; Sigma Chemical Company) was selected for generating ascites fluid (MzWH).

Polyclonal TPH antibodies (RzWH and SzWH, raised in rabbit and sheep, respectively) were purified from immune sera (pooled bleeds taken after maturation of the immune response) by affinity chromatography. The affinity matrix was made by coupling 2 mg of the full-length TPH purified by SDS-PAGE from inclusion bodies to 2 ml of AminoLink (Pierce) resin according to the manufacturer’s protocol, with the exception that 0.1% SDS was included in the pH 10.2 coupling buffer. Pooled sera were filtered (0.45 μm) and diluted with an equal volume of Tris phosphate buffered saline containing 0.2% (w/v) Nonidet P40, 20 mM benzamidine, 20 μM leupeptin prior to chromatography as previously described (Haycock, 1993a).

PH8, a mouse IgG1 monoclonal antibody that was generated against monkey liver phenylalanine hydroxylase (Jennings et al., 1986) but that also cross-reacts with TPH (Haan et al., 1987) was a gift from Dr I.G. Jennings and Dr R.G.H. Cotton (Parkville, Australia). The PH8 epitope (Cotton et al., 1988) is located within the sequence of phenylalanine hydroxylase that corresponds to residues 126–141 of rabbit TPH, a sequence that is entirely conserved in rabbit, rat, and human TPH (Grenett et al., 1987; Darmon et al., 1988; Boularand et al., 1990).

Working dilutions for each of the TPH antibodies were established empirically on the basis of antibody saturation curves in strip-blot immunolabeling assays of full-length recombinant TPH. The approximately ‘half-saturating’ working concentrations/dilutions used in subsequent blot immunolabeling experiments were as follows: PH8 and MzWH, 1:2000 from ascites fluid; RzWH, 1.0 μg/ml; SzWH, 0.5 μg/ml.

2.4. Immunohistochemistry

Tissue sections were incubated for 40–48 h at 4 °C in phosphate buffered saline (PBS) containing 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin, 3% normal cynomolgus serum (NcmS) and a mouse anti-TPH antibody (MzWH) at a dilution of 1:500. Sections were then incubated for 1 h at room temperature in PBS containing 0.3% Triton X-100, 3% NcmS and a 1:10000 dilution of peroxidase-rabbit anti-mouse IgG3 (Zymed Laboratories, San Francisco, CA) followed by a 1 h incubation in a PBS solution containing Vectastain (Vector Laboratories, Burlingame, CA) Elite ABC reagents and 0.3% Triton X-100. Finally, sections were washed in PBS and treated for 5 min with a solution of 0.05% 3,3′-diaminobenzidine (DAB) and 0.003% H2O2 dissolved in 0.03 M phosphate buffer. The DAB reaction product was intensified on slide-mounted sections with 0.005% osmium tetroxide (Condé et al., 1996) or 1.42% silver chloride (Pucak et al., 1996). Sections were then dehydrated and coverslips were applied with Permount.

2.5. Blot immunolabeling

Tissue samples were solubilized by sonication in 1% SDS, 5 mM Tris, 2 mM EDTA (final pH 8.0–8.3) and heating. Aliquots of the cellular and purified protein samples were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose sheets. Ponceau S staining was recorded xerographically and the transfers were destained/quenched in TPBS containing 1% polyvinylpyrrolidone and 0.05% Tween 20 (Haycock, 1993b). Transfers were then incubated (1 h, room temperature) sequentially with primary antibody (0.5–1.0 μg/ml), secondary antibody (0.6–0.8 μg/ml), and 125I-protein A (400–600 kcpm/ml; Amersham) with rinses in between each reagent (Haycock, 1993b). The secondary antibodies used were as follows: for PH8, rabbit anti-mouse IgG1 (DAKO); for MzWH, rabbit anti-mouse IgG3 (DAKO); for RzWH, swine anti-rabbit Ig (DAKO); for SzWH, rabbit anti-sheep IgG (H+L; Jackson ImmunoResearch). Immunoreactivity was visualized autoradiographically using X-ray film (XAR or BMS film; Kodak).

2.6. Peptide pre-competition

Peptides corresponding to sequences from rabbit, human, and rat TPH and from phenylalanine hydroxylase were synthesized by LSUMC Core Laboratories. All peptides were characterized by electrospray mass spectrometry, and relative purities were determined (80–90%) by analytical reversed-phase HPLC. For pre-competition studies, working dilutions of the TPH antibodies were pre-incubated with 1 μM peptide for 1 h at room temperature prior to incubation with strip-blots of purified full-length recombinant TPH.

3. Results

3.1. Immunohistochemistry

In immunohistochemical studies in monkeys, MzWH produced patterns of labeling corresponding to the known distribution of other markers of the serotonin system. For example, cell bodies and associated pro-
cessed in the DR nucleus were intensely immunoreactive (Fig. 1A). In addition, the laminar distribution and relative density of labeled axons in primary visual cortex (Fig. 1B) directly matched the innervation patterns previously demonstrated with antisera against 5-HT (Kosoksky et al., 1984; notably, the use of IgG3-specific secondary antibodies was necessary to achieve the level of reactivity illustrated in the figure). Cell bodies and associated processes in the DR nucleus were also labeled using SzWH as the primary antibody; however, even under ‘optimized’ conditions, only very weak cortical fibers staining was achieved (not shown).

MzWH, SzWH, and PH8 were compared in thaw-mounted and immersion-fixed cryosections from rapidly frozen midbrain blocks taken from autopsied brains. Each of the antibodies produced labeling of cell bodies and fibers within the DR, although the staining intensity achievable with SzWH was discernibly less than with either monoclonal antibody. Interestingly, under conditions optimal for the staining of DR cells, SzWH and PH8 also stained cell bodies in the substantia nigra whereas MzWH did not.

3.2. Monoclonal antibody epitope

In initial studies to determine the MzWH epitope, transfers prepared using a series of N-terminal deletion mutants of the recombinant rabbit TPH were analyzed. As shown in Fig. 2 (left panel), PH8 recognized equally full-length TPH and N-terminal deletion mutants of TPH (up to NA116), whereas MzWH recognized full-length TPH and NA60 TPH (as well as NA90 TPH; not shown) but not NA106 TPH or NA116 TPH, indicating that the MzWH epitope included amino acids between residues 90 and 106. A series of synthetic peptides were then used in antibody pre-competition studies to further resolve the residues involved in the MzWH epitope. As summarized in Table 1, the MzWH epitope is apparently located within the sequence E103SVPWF109 present in rabbit TPH. In addition, although the corresponding sequence in human and rodent TPH is ETVPWF, substitution of threonine for serine in one of the peptides only partially reduced the efficacy of its competition with MzWH reactivity with full-length recombinant rabbit TPH (Table 1; none of the peptides

Fig. 2. Immunoreactivity of monoclonal anti-TPH antibodies with full-length TPH and N-terminal TPH deletion mutants. Inclusion bodies from E. coli that expressed either full-length, NA60, NA106, or NA116 TPH were isolated and solubilized in SDS. The concentrations of TPH isoforms in the samples was estimated by Coomassie staining of SDS-PAGE gels, and two replicate sets of aliquots containing approximately 250 and 500 fmol subunit of each of the TPH isoforms were subjected to blot immunolabeling as described under Section 2.
Table 1  
Inhibition of TPH immunoreactivity by synthetic peptides

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<th>PEPTIDE SEQUENCE</th>
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<tr>
<td></td>
<td>MoWH</td>
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<td>TPH (Rb)</td>
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<td>PWFPKKIS</td>
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<tr>
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<td>GFDPKY</td>
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Aliquots of MoWH and PH8 antibody (1:2000 dilution from ascites fluid) were pre-incubated for 1 h with the indicated peptides (1 μM) immediately prior to incubation with strips from a single-well ‘trough’ blot of full-length recombinant TPH. Immunoreactivity was developed with rabbit anti-mouse IgG3 or IgG1 and 125I-protein A, visualized by autoradiography, and rated by visual inspection on an ordinal scale from ‘‘−’’ (no discernible immunoreactivity) to ‘+++’ (immunoreactivity in the absence of peptide).

![Fig. 3. Differential blot immunolabeling of rat and human DR and pineal gland (PIN) with anti-TPH antibodies. Four replicate sets of aliquots (~80 μg protein each) from the indicated samples were subjected to SDS-PAGE, transferred to a nitrocellulose sheet, and stained with Ponceau S. Individual sets were then quenched and incubated with the indicated primary antibody. Immunoreactivity was developed with appropriate secondary antibody and 125I-protein A and visualized by autoradiography. DR-, punches taken lateral to the DR.](image-url)
listed had a discernible effect upon the reactivities of SxWH or RzWH (data not shown)).

The PH8 epitope is located within the sequence of phenylalanine hydroxylase (Cotton et al., 1988) that corresponds to G126SELDAHPGFKDNVY141 in rat, rabbit, and human TPH. However, as also shown in Table 1, attempts to further delimit the PH8 epitope were unsuccessful.

3.3. Blot immunolabeling rat and human tissues

Fig. 3 summarizes the results of blot immunolabeling studies, using replicate aliquots of rat and human raphe and pineal extracts and the different anti-TPH preparations. TPH immunoreactivity from the four sources was present as a single 53–56 kDa band. PH8 also cross-reacted with a 58 kDa, non-TPH band in samples from human brain (Johansen et al., 1996; Stockmeier et al., 1996). Although TPH immunoreactivity from rat pineal gland, rat DR, and human DR migrated as a 56 kDa band, TPH immunoreactivity in human pineal gland from each of the four subjects migrated consistently as an approximately 3 kDa smaller band, as illustrated for one subject in Fig. 3 (note, although this difference is not as clear in the SxWH and RzWH autoradiographs shown, which were overexposed to reveal immunoreactivities (or lack thereof) with TPH in other tissues, the difference was apparent in shorter exposure autoradiographs). However, the relative intensities of immunolabeling of raphe versus pineal and rat versus human TPH differed among the antibodies. As compared with PH8 reactivities, MzWH was relatively raphe TPH-prefering, SxWH was relatively pineal TPH-prefering, and RzWH appeared to selectively recognize human pineal TPH (Fig. 3).

4. Discussion

Several different antibodies to TPH were developed as immunochemical reagents for the study of TPH in the CNS. The mouse monoclonal proved to be most robust for blot immunolabeling and in particular, for immunohistochemical analyses, as judged by its ability to stain not only cell bodies but fibers and neuropil in the cerebral cortex as well. Considering the relative conservation of this epitope across species (see below), it should prove equally useful in other mammalian species. Notably, this is the first monoclonal antibody raised against TPH.

As demonstrated in Fig. 3, both PH8 and SxWH recognized TPH from pineal gland and raphe, although to different degrees. On the other hand, MzWH and the polyclonal rabbit anti-TPH (RzWH) selectively detected a subset of TPH molecules. MzWH preferred raphe TPH, while RzWH preferred only human pineal gland TPH. Given that the coding regions of TPH mRNA from different tissues are reported to be the same but that the TPH proteins react differentially with the different antibodies, we report the use of new molecular tools which enable the differentiation of an enzyme which serves two disparate physiological roles. Whether or not this represents physical or structural differences in TPH correlating to physiological differences in biosynthetic pathway and/or protein stability/turnover remains to be determined.

Unlike PH8, which was raised against native phenylalanine hydroxylase purified from monkey liver (Jennings et al., 1986), the antibodies generated in the present studies were raised against full-length, SDS-denatured, recombinant rabbit TPH expressed in E. coli. Such bacterial antigens often lack post-translational modifications which are introduced in eukaryotic/mammalian expression systems, and the immune responses may have been biased toward such unmodified regions, particularly in the case of the autologous RzWH antibodies. To the extent that this may have occurred, differential reactivity of these antibodies with raphe TPH versus pineal gland TPH could reflect differential post-translational modification of such potential epitope(s) in vivo.

Based on results from peptide pre-competition studies, the epitope for the monoclonal MzWH was localized to the sequence ESVPWFP of rabbit TPH (and cognate ETVPWFP for rodent and human TPH). This sequence, however, does not reveal any site(s) that are obviously susceptible to enzymatic, post-translational modification. For example, although serine/threonine residues are potential phosphorylation sites, the surrounding residues do not create an obvious consensus recognition sequence for known protein kinases. One possible modification, oxidation of the Trp residue in the MzWH epitope, was suggested by the observation that storage of dried transfers at room temperature for several weeks substantially decreased the immunoreactivity of MzWH but not PH8, SxWH, or RzWH. Consistent with this possibility, storage of transfers at −20 °C between two sheets of plastic markedly reduced the time-dependent decrease in MzWH reactivity. Thus, it is possible that TPH in the pineal gland is more oxidized and that oxidation of the Trp residue in the MzWH epitope to a quinoline or other derivative contributes the lower relative immunoreactivity of MzWH with pineal versus raphe TPH, as compared with PH8.

Whereas a difference in isoelectric points has been reported for TPH from different tissues (Kim et al., 1991), much earlier reports of divergent structural differences (such as in apparent MWs) reflected comparisons across laboratories (cf. Nakata and Fujisawa, 1982). More recently (Dumas et al., 1989) and as demonstrated in the present study, TPH subunits from
rat raphe and pineal have been shown to comigrate as $M_t \approx 56000$ bands. TPH from human raphe also migrated as a 56 kDa band; however, the TPH band from human pineal gland was consistently $\sim 3$ kDa smaller than the other TPH bands. Chung et al. (2001) recently reported an equal abundance of both an upper and a lower TPH band in rat pineal extracts when using an unpurified rabbit antiserum to recombinant mouse TPH. However, as in the present study, they did not detect an upper band with other anti-TPH antibodies. That the lower $M_t$ TPH immunoreactivity in human pineal extracts was observed with PH8, affinity-purified SzWH, and affinity-purified RzWH supports its identity as TPH. That this lower $M_t$ species resulted from proteolysis postmortem or during sample storage/processing seems unlikely for several reasons. First, the TPH immunoreactive band from each of four pineal glands migrated as a 53 kDa band, and 56 kDa reactivity was not discernible in any of these samples despite a wide range of postmortem intervals (9.5–27 h). Second, 53 kDa reactivity was not discernible in the DR samples from the same subjects with any of the antibodies. Third, neither of the polyclonal antibodies revealed any evidence of a lower $M_r$ ‘proteolytic ladder’—which is characteristic of ‘endogenous’ proteolysis in postmortem human samples—in any of the human pineal glands. An alternative explanation, suggested by the discovery of a TPH cDNA splice variant in human but not rat tissues (Wang et al., 1998), is that an as yet to be described alternative splicing variant is predominantly translated in human pineal. That is, although the abundance of the newly described variant relative to the ‘normal’ differed among brain regions, its translation product is predicted to be approximately 2.5 kDa higher as opposed to lower than the ‘normal’ translation product.

In conclusion, although the primary sequences of rat, human, and monkey TPH are very similar and those of pineal gland and raphe TPH proteins within a species are identical, the immunochemical differences reported in the present study indicate that there are substantial immunochemical differences among TPH proteins in situ which may reflect differences in their physical and/or physiological state.

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