Molecular cloning and nucleotide sequence of a cDNA encoding hydroxyindole *O*-methyltransferase from chicken pineal gland

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Hydroxyindole *O*-methyltransferase (EC 2.1.1.4) is the enzyme that catalyses the synthesis of melatonin in the pineal gland and in the retina. Polyadenylated RNA from chicken pineal glands was used to prepare a cDNA library in λ gt11. The library was screened with an antiserum directed against chicken hydroxyindole *O*-methyltransferase, and one cDNA clone was isolated. The fusion protein expressed by phage lysogens was identified on Western blots as a 165 kDa immunoreactive protein (β -galactosidase, 110 kDa; hydroxyindole *O*-methyltransferase, 38 kDa). The fusion protein exhibited hydroxyindole *O*-methyltransferase activity. Its K_m values for *N*-acetyl-5-hydroxytryptamine and *S*-adenosylmethionine were 5 times those of the natural enzyme. The intrinsic activity of the fusion protein was approx. 0.25% that of the natural enzyme. The cDNA consisted of 1436 nucleotides, including a 1038-nucleotide sequence encoding a full-length 346-amino-acid hydroxyindole *O*-methyltransferase. Comparison with bovine hydroxyindole *O*-methyltransferase [Ishida, Obinata & Deguchi (1987) *J. Biol. Chem.* **262**, 2895–2899] revealed 52% identity in nucleotide sequences and 44% identity in peptide sequences. Northern-blot analysis revealed the presence of hydroxyindole *O*-methyltransferase mRNA transcripts in chicken pineal gland and retina, but not in the telencephalon.

INTRODUCTION

Experimental evidence obtained in different species of mammals and birds has indicated that the indolic hormone melatonin, produced in the pineal gland, plays a central role in controlling seasonal breeding and circadian activity/rest cycles (Zimmerman & Menaker, 1979; Reiter, 1980). In some species, it has been shown that melatonin is also produced in the retina, where it appears to regulate disc shedding from rod photoreceptors and dopamine release from amacrine cells (Besharse & Dunis, 1983; Dubocovich, 1983). A better understanding of the regulation of melatonin production in the pineal gland and in the retina would require investigations at the molecular level on the enzymes involved in the melatonin pathway. Hydroxyindole *O*-methyltransferase (HIOMT, EC 2.1.1.4) catalyses the terminal step of melatonin synthesis by transferring a methyl group from *S*-adenosylmethionine to the 5-hydroxy group of *N*-acetyl-5-hydroxytryptamine (Axelrod & Weissbach, 1961). Previous studies in mammals and birds have indicated that HIOMT activity in the pineal gland can double or halve over several days, if the animals are kept in constant light or in constant darkness (Wurtman *et al.*, 1963; Lauber *et al.*, 1968; Yang & Neff, 1976). The molecular basis of this regulation of HIOMT expression remains ill-defined, and there is virtually no information available on the regulation of HIOMT in the retina. The switch-on of the HIOMT gene during embryonic life constitutes a limiting step in the differentiation of the melatoninergic phenotype in the pineal gland and retina. The mechanism of this cell-specific expression of the enzyme during development requires further investigation. The molecular cloning of a cDNA encoding HIOMT is a prerequisite to examine the transcriptional regulation of this enzyme. A previous study has reported the sequence of a cDNA encoding HIOMT from bovine pineal gland (Ishida *et al.*, 1987).

However, RNA-blot analysis revealed no HIOMT mRNA transcript in the bovine retina (Ishida *et al.*, 1987). This was consonant with other studies reporting a lack of HIOMT immunoreactivity in the bovine retina (Kuwano *et al.*, 1983). Therefore it appeared interesting to clone HIOMT cDNA in the chicken, because in this species HIOMT immunoreactivity can be detected in both the pineal gland and the retina (Voisin *et al.*, 1988). In addition, such a study would provide information on evolutionary changes in the structure of HIOMT. In the present paper, we describe the molecular cloning of a cDNA encoding chicken HIOMT. This cDNA probe hybridizes with HIOMT mRNA transcripts from chicken pineal gland and retina. Comparison of nucleotide and peptide sequences reveals about 50% identity with bovine HIOMT.

MATERIALS AND METHODS

Antibodies

The antibodies directed against chicken HIOMT have been characterized in previous studies (Voisin *et al.*, 1988; Guerlotti *et al.*, 1988). Briefly, an antiserum was obtained in a rabbit immunized with a 30%-pure preparation of HIOMT. The antiserum was then purified by two rounds of adsorption on chicken brain proteins. This purified antiserum was used to screen the chicken pineal cDNA library. To ensure that the immunostaining of the fusion protein on Western blots was not due to multiple reactivities of the antiserum, the experiment was performed with affinity-purified anti-HIOMT immunoglobulins. These were obtained by incubating the antiserum with the HIOMT protein band separated on a Western blot, followed by desorption of the immunoglobulins with a pH shock (Guerlotti *et al.*, 1988). Goat serum anti-rabbit γ -immunoglobulins (GAR; Sigma R-5506) and peroxidase–rabbit anti-

Abbreviations used: HIOMT, hydroxyindole *O*-methyltransferase; poly(A)⁺ RNA, polyadenylated RNA.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

peroxidase complex (PAP; Sigma P-1291) were used for the immunochemical stain. All the immunochemical procedures were performed in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) containing 0.05% Tween (PBS/Tween).

Isolation of total and polyadenylated [poly(A)⁺] RNA

Tissues were homogenized in 4 M-guanidine isothiocyanate and centrifuged at 130 000 *g* for 18 h through a 5.7 M-CsCl pad (Chirgwin *et al.*, 1979). The pellet of total RNA was resuspended, phenol-extracted and ethanol-precipitated. Poly(A)⁺ RNA was prepared by affinity chromatography on oligo(dT)-cellulose columns, by using a commercially available kit (Pharmacia).

Construction of a cDNA library

Chicken pineal poly(A)⁺ RNA (2 µg) was used as template to synthesize single-stranded cDNA by extending oligo(dT) primers with reverse transcriptase (Amersham). Double-stranded cDNA was obtained by adding RNAase H and DNA polymerase I and cloned in the λgt11 vector, by using *Eco*RI/*Bam*HI adaptors (Amersham). The recombinant phages (3 × 10⁵) were amplified and screened on *Escherichia coli* Y1090 (r-).

Screening of phage library

The λgt11 cDNA library was screened for the expression of a fusion protein comprising antigenic moieties of HIOMT as described by Mierendorf *et al.* (1987). The nitrocellulose replicas of the bacterial clones infected with the λgt11 cDNA library were blocked with 2% gelatin and 1% BSA and incubated overnight in anti-HIOMT antiserum diluted 1/1000. The immunochemical staining was performed by sequential incubations (1 h each) in GAR diluted 1/100 and PAP diluted 1/200, followed by colour development with 0.05% diaminobenzidine and 0.03% H₂O₂.

Characterization of the fusion protein

λgt11-HIOMT phage lysogen was obtained by infection of *E. coli* Y1089 (r-) and selection for temperature-dependent lysis, as described by Huynh *et al.* (1985). An infected clone was grown at 32 °C in 100 ml until *A*₆₀₀ = 0.6. The culture was shifted to 44 °C for 20 min, 10 mM-isopropyl β-D-thiogalactoside was added and the culture was further incubated at 38 °C for 60 min. Bacteria were collected by centrifugation (1000 *g* for 20 min), resuspended in 0.02 vol. of 50 mM-phosphate buffer, pH 7.9, and frozen in liquid nitrogen. After sonication for 15 s at 4 °C, the homogenate was centrifuged at 10 000 *g* for 15 min at 4 °C and the supernatant (5 mg of protein/ml) was used as the source of fusion protein. Samples containing 50 µg of protein were analysed by SDS/PAGE and transferred to nitrocellulose sheets. Western blots were blocked with 2% gelatin and 1% BSA and incubated overnight with a solution of affinity-purified anti-HIOMT immunoglobulins corresponding to a 1/2000 dilution of the antiserum (as estimated on Western blots of pineal HIOMT). Immunochemical staining with GAR and PAP was performed as described above. HIOMT activity was assayed in a final volume of 100 µl containing 50 mM-sodium phosphate, 2–200 mM-*N*-acetyl-5-hydroxytryptamine, 10–200 µM-*S*-adenosyl[methyl-³H]methionine (NEN; final sp. radioactivity 25 Ci/mol) and 10–250 µg of protein. Blanks were assayed without protein or without *N*-acetyl-5-hydroxytryptamine. Incubation was for 30 min at 37 °C, followed by chloroform extraction of the reaction product, as previously described (Voisin *et al.*, 1988). Occasionally, the chloroform extract was evaporated, the reaction product was dissolved in 100 µl of ethanol containing 10 µg of authentic melatonin and analysed by t.l.c. on a silica-gel plate with chloroform/methanol/acetic acid

Fig. 1. Western-blot analysis of fusion protein and chicken pineal HIOMT

Proteins were separated by SDS/PAGE, transferred to a nitrocellulose sheet and immunostained with affinity-purified anti-HIOMT immunoglobulins: (a) 50 µg of pineal proteins separated on 12.5% acrylamide; (b) 50 µg of proteins from λ-HIOMT-infected *E. coli* Y1089 (r-) separated on 12.5% acrylamide; (c) 50 µg of proteins from λ-HIOMT-infected *E. coli* Y1089 (r-) separated on 7.5% acrylamide.

(90:10:1, by vol.), as previously described (Klein & Notides, 1969).

DNA sequencing

The recombinant λgt11 HIOMT phage clone was digested with the restriction enzyme *Bam*HI, which cut the adaptors used for cDNA cloning and released the full-length 1.5 kb HIOMT cDNA insert. The insert was then digested with restriction endonucleases *Eco*RI, *Hind*III, *Pst*I, *Pvu*II and *Rsa*I and subcloned into the mp 18 and mp 19 M13 phage vectors. Dideoxy sequencing reactions (Sanger *et al.*, 1977) were performed with T7 DNA polymerase and deaza-dGTP by using fluorescent primers, and analysed on an automated laser fluorescent DNA sequencer (Pharmacia).

Northern-blot analysis

Total and poly(A)⁺ RNAs were analysed on 1% agarose/0.7 M-formaldehyde gels and transferred to nitrocellulose sheets (Davis *et al.*, 1986). The cDNA probe used for hybridization was the 1.5 kb *Bam*HI insert, radioactively labelled by random priming. Hybridization and washing under high stringency conditions (1 × SSC, 42 °C) were as previously described (Cogné *et al.*, 1988).

RESULTS

Cloning of HIOMT

Out of 2 × 10⁵ recombinant phages, one HIOMT-positive clone (referred to as λ-HIOMT) was isolated and purified by two additional rounds of screening. The size of the cDNA insert was approx. 1.5 kb. The fusion protein expressed by phage lysogens

Fig. 2. Enzyme activities of fusion protein and chicken pineal HIOMT

(a) Extracts from λ -HIOMT-infected (λ -HIOMT) or non-infected (control) *E. coli* Y1089 (r-) (50 μg of protein) were assayed for HIOMT activity with 100 μM -*S*-adenosyl[*Me*- ^3H]methionine, with (■) or without (□) 100 μM -*N*-acetyl-5-hydroxytryptamine (NAS) as acceptor substrate. Blanks were assayed with both substrates in the absence of proteins. (b) HIOMT activity was assayed with increasing amounts of proteins from λ -HIOMT-infected *E. coli*. Both substrates were at 100 μM concentration. (c) and (d) HIOMT activity was assayed with 250 μg of proteins from λ -HIOMT-infected *E. coli* (c) or with 5 μg of chicken pineal proteins (d). The concentrations of NAS (●—●) and *S*-adenosylmethionine (SAM; ■—■) were varied as indicated. The corresponding fixed substrate was at 100 μM concentration.

was analysed on Western blots (Fig. 1). Positive immunochemical reactions were obtained with an affinity-purified antibody directed against chicken HIOMT. The apparent molecular mass of the fusion protein (165 kDa) was approximately the sum of the molecular masses of β -galactosidase (110 kDa) and HIOMT (38 kDa). As estimated from the intensity of the immunochemical staining, the concentration of the fusion protein in lysates of λ -HIOMT-infected *E. coli* was comparable with the concentration of HIOMT in chicken pineal homogenates (Fig. 1). The fusion protein catalysed the transfer of a [^3H]methyl group from *S*-

adenosylmethionine to *N*-acetyl-5-hydroxytryptamine (Fig. 2). The radioactive product co-migrated with authentic melatonin on t.l.c. (results not shown). Non-specific reactions estimated in the absence of enzyme or in the absence of *N*-acetyl-5-hydroxytryptamine represented about 15% of the total (Fig. 2). No HIOMT activity was detected in homogenates of non-infected *E. coli* (Fig. 2). The reaction product increased linearly with increasing amounts of fusion protein (Fig. 2). The kinetics of fusion HIOMT and natural HIOMT were compared (Fig. 2). Apparent K_m values for both *N*-acetyl-5-hydroxytryptamine and *S*-adenosylmethionine were approx. 5 times higher for fusion HIOMT than for chicken pineal HIOMT. The apparent V_{max} of fusion HIOMT was about 0.25% that of pineal HIOMT, when V_{max} values were expressed per mg of protein (Fig. 2). This result may reflect different intrinsic activities of the enzymes, as the concentration of fusion HIOMT in *E. coli* extracts compared well with that of natural HIOMT in pineal homogenates (see Fig. 1). Fusion HIOMT also differed from pineal HIOMT in that it appeared insensitive to inhibition by high concentrations of *N*-acetyl-5-hydroxytryptamine (Fig. 2).

Nucleotide sequence of chicken HIOMT cDNA

The complete sequence of the 1436 bp HIOMT cDNA has been determined in both orientations (sequence and restriction map on Fig. 3). In addition, a 1 kb *Pvu*II fragment encompassing part of the *lacZ* vector sequence and part of the cDNA insert has been studied, in order to determine the orientation and the reading frame in which the HIOMT sequence was translated. The sequence began with an ATG codon at position +3 which, by comparison with the sequence of bovine HIOMT (Fig. 3), is likely to correspond to the initiation codon of the HIOMT mRNA. Sequence +3 to +1040 encoded a 346-amino-acid protein. Bovine and chicken nucleotide sequences displayed 52% identity in the coding region, and peptide sequences were 44% identical (Fig. 3). The translation termination codon was followed by an untranslated sequence that contained the polyadenylation signal AATAAA about 30 nucleotides before the end of the cDNA.

RNA-blot analysis

A probe of λ -HIOMT cDNA was used to analyse RNA from chicken pineal gland, retina and telencephalon, under high stringency conditions. Northern-blot analysis of both total and poly(A)⁺ RNA from pineal gland revealed a single band of approx. 1.8 kb (Fig. 4). The detection limit was 0.2 μg of pineal total RNA (Fig. 4). HIOMT mRNA transcripts of 1.8 kb were also detected in retinal poly(A)⁺ RNA, but not in retinal total RNA (Fig. 4). Densitometric analysis of the autoradiographs indicated that radioactive labelling was about 30-fold higher on pineal than on retinal poly(A)⁺ RNA. No hybridization could be observed on either total or poly(A)⁺ RNA from telencephalon (results not shown).

DISCUSSION

A cDNA encoding chicken HIOMT has been cloned in $\lambda\text{gt}11$ and characterized. The β -galactosidase-HIOMT fusion protein expressed by phage lysogens exhibited HIOMT activity, thus allowing positive identification of the clone. Peptide-sequence similarity between bovine HIOMT (Ishida *et al.*, 1987) and chicken HIOMT (44% identity) points to a medium rate of evolution of the protein (approx. 20 point mutations per 100 amino acids per 10^8 years), based on the criteria presented by Creighton (1984). Chicken HIOMT also displayed 24% identity with hydroxyneurosporene *O*-methyltransferase (Armstrong *et al.*, 1989), an enzyme involved in the biosynthesis of carotenoids

Fig. 4. Northern-blot analysis of pineal and retinal RNAs

The indicated amounts of total or poly(A)⁺ RNA from pineal gland or retina were analysed on 1% agarose/0.7 M-formaldehyde gels, transferred on to nitrocellulose and probed with ³²P-labelled λ -HIOMT cDNA (*Bam*HI fragment). Size markers on the left panel correspond to large (5 kb) and small (2 kb) rRNAs.

in *Rhodobacter*. Comparison of HIOMT with the methyltransferases involved in catecholamine metabolism revealed few similarities: peptide sequence identity was 8% with human phenylethanolamine *N*-methyltransferase (PNMT; Kaneda *et al.*, 1988) and only 3% with human catechol *O*-methyltransferase (COMT; Bertocci *et al.*, 1991). However, a region of chicken HIOMT between Lys-262 and Leu-287 displayed a concentration of peptide identities with bovine HIOMT (69%), neurosporene *O*-methyltransferase (42%), PNMT (50%) and COMT (31%). Site-directed-mutation experiments should decide whether this region of higher conservation is important for the catalytic activity of the enzyme. In different animal species, including chicken, long-term effects of constant light or constant darkness on HIOMT activity have revealed that this enzyme is controlled by environmental lighting (Wurtman *et al.*, 1963; Lauber *et al.*, 1968). In some species of seasonal breeders, changes in HIOMT activity appear to be correlated with seasonal changes in the reproductive status (Barfuss & Ellis, 1971; Preslock, 1974; Ellis & Balph, 1976). The cDNA probe characterized in the present study should allow us to determine whether HIOMT expression in the chicken pineal gland is regulated at the transcriptional level and to identify the hormones and second messengers involved in this process. Another interesting line of research will be to examine HIOMT expression in the retina. Indeed, our experiments performed in the chicken are the first to show the

presence of HIOMT mRNA transcripts in the retina. Hybridization of the HIOMT cDNA probe *in situ* in the retina should be of special interest, because previous attempts to localize HIOMT in this tissue have yielded conflicting results. To limit the discussion to the bovine retina, some authors have reported the presence of a 25 kDa HIOMT-like protein in retinal photoreceptors (Wiechmann *et al.*, 1985), whereas other authors have independently reported a lack of HIOMT immunoreactivity (Kuwano *et al.*, 1983) and absence of mRNA transcripts (Ishida *et al.*, 1987). The situation is clearer in the chicken, where HIOMT protein and mRNA transcripts are of identical size in the retina and in the pineal gland (Voisin *et al.*, 1988; the present work). The relatively high level of HIOMT expression in the chicken retina, as compared with mammals, should facilitate further studies on this tissue. The existence of a regulatory feedback loop between the melatoninergic and dopaminergic systems of the retina has been documented (Pierce & Besharse, 1986). In the chicken retina, melatonin is known to inhibit dopamine release (Dubocovich, 1984), whereas light and dopamine inhibit melatonin synthesis at the 5-hydroxytryptamine-acetylation step (Hamm & Menaker, 1980; Zawilska & Iuvone, 1989). It is not known whether HIOMT is also regulated in this tissue. Further studies of HIOMT expression using antibodies and cDNA probes should provide a better understanding of the melatoninergic function of the retina.

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Fig. 3. Nucleotide sequence of chicken HIOMT cDNA

(a) Restriction map of the cDNA and sequence strategy. The protein coding region is indicated by the shaded box. The arrows indicate the direction and extent of sequence determination. (b) Nucleotide sequence and deduced amino acid sequence of chicken HIOMT. The termination codon is noted with a star and the polyadenylation signal in the 3' non-coding region is underlined. (c) Comparison of the amino acid sequences of chicken HIOMT and bovine HIOMT (Ishida *et al.*, 1987). Identical amino acids are noted with a star. The hyphen indicates a gap introduced for optimal alignment.

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