Immunological detection of phenylalanine hydroxylase protein in *Drosophila melanogaster*

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A monoclonal antibody raised against monkey liver phenylalanine hydroxylase (PAH) has been used to detect this protein in *Drosophila melanogaster*. A cross-reacting material (CRM) band of apparent molecular mass 50–52 kDa, equivalent to that deduced for the *Drosophila melanogaster* PAH protein based on the *pah* gene cDNA sequence, has been detected. This CRM was analysed throughout development and showed an equivalent pattern to that reported for PAH activity in this insect, with maxima at pupariation and at pharate adult formation. Distribution of this CRM in larval tissues, the haemolymph and the adult body is mainly restricted to the larval fat body and the adult head. Demonstration of this CRM as the PAH protein comes from the correlation between the decreased PAH enzyme activities of two mutant strains and their decreased amounts of CRM by Western blotting.

INTRODUCTION

Phenylalanine hydroxylase (PAH; EC 1.14.16.1) is the enzyme that catalyses the formation of L-tyrosine from L-phenylalanine using a tetrahydropterin as cofactor. This enzyme is one member of a family of aromatic amino acid hydroxylases including tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4). All three hydroxylases are closely related in structure and function (Ledley *et al.*, 1985; Grenett *et al.*, 1987).

Mammalian PAH is a rather complex enzyme, susceptible to regulation by many effector molecules (phenylalanine, tetrahydrobiopterin, etc.). It is thought that the reaction that it catalyses is the rate-limiting step in the degradation of phenylalanine, the accumulation of which produces severe disorders in brain development as well as other characteristic clinical symptoms in man (Scriver & Clow, 1980).

In contrast to the situation found in mammals, very few reports have appeared on insect PAH (Geltosky & Mitchell, 1980; Bel & Ferré, 1989; Bel *et al.*, 1990) and thus very little is known about this enzyme in insects. In fact, there is still controversy as to whether tryptophan hydroxylase is present in *Drosophila* or, on the contrary, whether the conversion of tryptophan to 5-hydroxytryptophan is performed by some of the other hydroxylases of the family (Neckameyer & White, 1989).

PAH proteins from different organisms share important structural similarities. This has been brought to light by studies with monoclonal antibodies, in which antibody PH8 against monkey PAH reacted with PAH from many species, from human to fish (Cotton *et al.*, 1988). This monoclonal antibody recognizes an epitope localized between residues 139 and 154 of the enzyme (Cotton *et al.*, 1988). The extremely good conservation of this region makes this antibody useful for the detection of PAH as well as other aromatic amino acid hydroxylases in a wide range of species.

In the present paper we describe the application of this antibody to the detection of PAH in *Drosophila*, demonstrating that the cross-reacting material (CRM) detected is indeed the

PAH protein. We also describe the developmental and tissue distribution of this protein.

EXPERIMENTAL

Drosophila culture

Flies were maintained at 25 °C in bottles with 30 ml of a standard medium of corn flour, agar, sugar and live yeast. Three strains were used in the experiments: a wild-type strain (*Oregon R*), and two *Henna* mutant strains (*ju Hn^r h* and *Hn^r sr*). All the strains were obtained from the *Drosophila* Stock Center (Bowling Green, OH, U.S.A.).

PH8 antibody purification

The isolation and characteristics of the PH8 monoclonal antibody [mouse anti-(monkey PAH)] have been described by Jennings *et al.* (1986). Ascites fluid containing PH8 antibody was purified through a Staphylococcal Protein A–Sephadex column by using the procedure described by Choo *et al.* (1981).

Sample preparation for electrophoresis

Two insects (larvae, pupae or adults) were homogenized in 50 µl of a solution containing 0.05 M-Tris/HCl, pH 6.8, 1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol, and centrifuged at 15600 *g* for 5 min at 4 °C. Then the supernatant was mixed with a concentrated sample buffer in order that the final concentrations were those of Laemmli sample buffer (Laemmli, 1970). Samples were not directly homogenized in Laemmli buffer since, in larval extracts, part of the protein content was lost during centrifugation. Aliquots of the supernatant (15 µl) were used to load the gel. The amount of protein loaded in each well was equivalent to one-tenth of an insect (larva, pupa or adult).

An equivalent protocol was employed for tissues or body parts. Five heads or thorax/abdomens from adult flies were homogenized in order to obtain concentrated extracts (the amount loaded was equivalent to one-half of the protein content of a head or a thorax/abdomen). Larval tissues (six of each) were

Abbreviations used: PAH, phenylalanine hydroxylase; *pah*, gene coding for PAH protein; PBS, phosphate-buffered saline (0.01 M-sodium phosphate/0.13 M-NaCl, pH 7.5); CRM, cross-reacting material.

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collected after dissection in phosphate-buffered saline (PBS). Haemolymph was obtained from eight larvae that had been punctured with a needle, layered on a glass wool pellet in a microcentrifuge tube and centrifuged at 15600 *g* for 5 min. The haemolymph was obtained at the bottom of the tube.

Western blot analyses

SDS/PAGE was carried out using the discontinuous buffer system (Laemmli, 1970). After electrophoresis at 70 V overnight, semi-dry electroblotting on to a nitrocellulose sheet (Schleicher & Schuell, BA85, 0.45 μ m) was carried out for 1 h with a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia).

Filters were blocked in 3% (w/v) BSA in PBS for 1 h at room temperature with continuous rocking, and then incubated overnight at room temperature with purified PH8 monoclonal antibody diluted at 1:25000 in 1% (w/v) BSA in PBS. Then the filter was washed several times in 0.1% (v/v) Tween-80 in PBS for a period of 2 h. Second antibody incubation was carried out with peroxidase-conjugated goat anti-mouse IgG antibody (Immunotech, Marseille, France) diluted at 1:5000 in 1% (w/v) BSA in PBS for 1 h. After several washes with 0.1% (v/v) Tween-80 in PBS, a peroxidase colour reaction was performed (Saiga *et al.*, 1987).

Blanks with no PH8 antibody were run with either total mouse IgG (Sigma) or mouse ascites fluid without anti-PAH antibodies.

Purification of rat liver PAH

Rat liver PAH was purified by substrate-induced hydrophobic chromatography according to published procedures (Shiman *et al.*, 1979).

PAH activity

Samples to be analysed were prepared by homogenizing 1 g of frozen third-instar larvae in 4 ml of 0.1 M-KH₂PO₄ buffer (pH 7.0) containing 3 mM-dithiothreitol. The homogenate was centrifuged at 27000 *g* for 20 min at 4 °C and the supernatant was filtered through a double layer of gauze.

PAH activity was measured by the conversion of [¹⁴C]phenylalanine into [¹⁴C]tyrosine according to the method of Ledley *et al.* (1987), with 0.6 mM-[U-¹⁴C]phenylalanine (specific radioactivity 1.78 mCi/mmol) and 0.18 mM-tetrahydrobiopterin. Tubes were incubated for 10 min at 25 °C under dim red light. [¹⁴C]Phenylalanine and [¹⁴C]tyrosine were separated by t.l.c. using chloroform/methanol/NH₃ (11:7:2, by vol.). Radioactivity was measured on the plates with a radio-t.l.c.-analyser (RITA) from Raytest (Straubenhardt, Germany), with Argon/10% methane as counting gas. Because of the instability of the enzyme upon freezing, only fresh extracts were used. Protein was assayed by the method of Bradford (1976), using BSA as standard.

RESULTS

Identification of protein electrophoretic bands binding the PH8 antibody

To determine if PH8 could recognize PAH or other aromatic amino acid hydroxylases from *Drosophila melanogaster*, electrophoresis on an SDS/10%-polyacrylamide gel was carried out. The separated proteins were transferred to a nitrocellulose filter and incubated with PH8 antibody (Fig. 1). Purified rat liver PAH was used as a positive control. As negative controls, anti-PAH antibody was replaced by ascites fluid not containing anti-PAH antibodies (Fig. 1a) and by total mouse IgG (Fig. 1b). In *Drosophila* pupae samples, only one protein band stained strongly

(Fig. 1, lanes 9 and 10). This band migrated exactly as did purified rat PAH (Fig. 1, lane 7), with an apparent molecular mass of 50–52 kDa. This CRM was only present in white and dark pupae, but not in adult flies (more than 4 days after emergence) (Fig. 1, lane 8).

The specificity of the binding reaction was confirmed by the negative controls. The two control antibody solutions were used at low dilution in order to detect just those protein bands that presented higher affinities for antibodies or that were at higher concentrations in the test samples. The binding patterns with these negative controls (Figs. 1a and 1b) were similar to that obtained with Coomassie Blue (results not shown). Neither negative control gave a significant signal in the 50–52 kDa region, where PAH migrates, confirming the specificity of binding of this band to the PH8 antibody (Fig. 1c).

Fig. 1. Western blot analysis of PH8 antibody cross-reactive bands in *Drosophila* extracts after SDS/PAGE

Drosophila total proteins were separated in SDS/PAGE and, after blotting, were incubated with (a) ascites fluid not containing anti-PAH antibodies, (b) total mouse IgG, or (c) PH8 antibody. The runs represent crude extracts of white pupae (lane 10), dark pupae (lanes 2, 5 and 9), adult flies (lane 8), purified rat PAH (lanes 1, 4 and 7), and low-molecular-mass markers (Bio-Rad) (lanes 3 and 6). Electrophoretic mobilities of the markers are indicated at the right and correspond to molecular masses of 97, 66, 45, 31 and 21 kDa from top to bottom.

Fig. 2. Developmental analysis of PAH CRM from wild-type *Drosophila melanogaster* by Western blot analysis after SDS/PAGE

Lane 1, late third instar larvae; 2, white pupae; 3, 1-day-old pupae; 4, pharate adult; 5, 0–4-h-old adult; 6, 4–5-day-old adult. Lanes 7 and 8 contain concentrated extracts ($\times 5$) of heads and thorax/abdomens respectively from 0–1-day old adult flies.

The mass of the putative PAH protein detected by our experiments agrees very well with that deduced for the PAH protein of *Drosophila melanogaster* (51.9 kDa) based on the *Drosophila pah* gene cDNA sequence (Morales *et al.*, 1990), and is different from the value of 58 kDa reported for *Drosophila* tyrosine hydroxylase (Neckameyer & Quinn, 1989).

Developmental pattern of the putative PAH protein

To determine the developmental pattern of this protein, Western blots were carried out with samples of insects at different stages of development. The results in Fig. 2 show that the putative PAH CRM is present from at least the third larval instar until early adult life. The strongest signal is found at pupariation (Fig. 2, lane 2) with a second smaller peak at the end of the pupal stage, when a pharate adult is formed (Fig. 2, lane 4). From these results and those from other Western blot analyses, it can be seen that the amount of CRM rapidly decreases after emergence of the adult, becoming practically undetectable after 4 days (Fig. 2, lane 6).

In thorax/abdomen extracts and in old flies, two or three bands of lower molecular mass than PAH cross-reacted with the antibody (Fig. 2, lanes 6 and 8). These bands could be either the product of unspecific binding or PAH degradation products. The former is more likely, since abdomens of females contain large amounts of yolk proteins, which are revealed in Coomassie Blue-stained gels as thick bands of the same molecular mass as those that cross-react with the antibody (results not shown).

Tissue specificity of the putative PAH protein in larvae

An analysis of the tissue localization of the 50–52 kDa cross-reacting protein was carried out with three organs from late third instar larvae (salivary glands, fat body and brain) and with larval haemolymph (Fig. 3). The 50–52 kDa protein was restricted to fat body extracts, which agrees well with the fact that this tissue is the only one that contains PAH mRNA in *Drosophila melanogaster* (Morales *et al.*, 1990).

In young adults the PAH protein is also distributed in a tissue-specific manner, since it is mainly detected in the head (see Fig. 2), and is probably located specifically in the head fat body.

Correlation between PAH enzyme activity and amount of CRM in Western blots

Direct evidence for the identity of this CRM as PAH protein

Fig. 3. Tissue distribution of PAH in *Drosophila* larvae as revealed by Western blotting after SDS/PAGE
Lane 1, salivary glands; 2, brain; 3, fat body; 4, larval haemolymph; 5, whole larva; 6, whole white pupa.

Table 1. PAH activity in *Drosophila* strains

Values represent the means $\pm$ S.E.M. of five independent measurements for the wild-type strain (*Or-R*) and of three measurements for the two *Henna* mutant strains (*Hn^{r3}* and *Hn^r*). Activity was measured in third instar larvae close to pupariation. PAH activity is expressed as pmol of tyrosine formed/min per mg of protein.

Strain	PAH activity	
	(pmol/min per mg)	(% of wild-type)
<i>Or-R</i>	2591 $\pm$ 136	100
<i>Hn^{r3}</i>	247 $\pm$ 57	9.5
<i>Hn^r</i>	715 $\pm$ 190	27.6

Fig. 4. PAH CRM in *Henna* mutant strains
Western blotting after SDS/PAGE of adult heads (lanes 1–6) and white pupae (lanes 7–11). Wild-type strain *Oregon R* (lanes 3, 6 and 9), *Hn^{r3}* strain (lanes 2, 5, 8 and 11) and *Hn^r* strain (lanes 1, 4, 7 and 10) were used.

was obtained by a comparison of PAH activity and the amount of CRM in the wild-type and two *Henna* strains showing mutations of the putative *pah* gene (P. Ruiz-Vázquez, Y. Bel, J. Ferré and F. J. Silva, unpublished work). Both strains showed decreased PAH enzyme activity (Table 1) which was correlated with the amounts of CRM detected by Western blots (Fig. 4). The more defective strain (*Hn^{r3}*) showed only traces of both PAH enzyme activity and PAH protein, whereas *Hn^r* showed intermediate amounts of CRM and enzyme activity.

DISCUSSION

PAH is a protein that has been well conserved throughout evolution. The degree of similarity in amino acid composition between humans and rats is very high (92%) (Dahl & Mercer, 1986), and between humans and *Drosophila* it is around 55% (Morales *et al.*, 1990). The long central part of the protein where the catalytic domain lies (Grenett *et al.*, 1987) is even better conserved, with similarities of around 80% between humans and *Drosophila melanogaster*.

PH8 antibody recognizes an epitope located in this long central part of PAH, in a region from residues 139 to 154 (Cotton *et al.*, 1988). This epitope seems to be well conserved not only between PAH proteins of various species, but also among the

three aromatic amino acid hydroxylases (Grenett *et al.*, 1987). In *Drosophila melanogaster*, only three out of 16 amino acids are not conserved in this region compared to the mammalian PAH protein (Morales *et al.*, 1990), whereas the tyrosine hydroxylase protein differs in seven out of 16 amino acids (Neckameyer & Quinn, 1989).

Cotton *et al.* (1988) have previously described the immuno-reactivity of this antibody with one or two bands of around 50 kDa in Western blots of electrophoretically separated proteins from liver extracts of several vertebrate species. This suggests that this antibody could be used for the detection of aromatic amino acid hydroxylases in a wide range of species.

The fact that only three out of 16 amino acids are not conserved in the region of the *Drosophila* PAH protein which comprises the PH8 epitope provided a good indication that PH8 could be used for the immunological detection of PAH in this insect. This was less likely for tyrosine hydroxylase, since the degree of similarity was lower, and also because Haan *et al.* (1987) have reported that PH8 does not bind partially purified tyrosine hydroxylase from bovine adrenal gland in Western blots.

Western blots after SDS/PAGE from *Drosophila* extracts have shown that a reactive band is detected with this PH8. The estimated molecular mass (50–52 kDa) agrees with the one deduced for the PAH protein of *Drosophila melanogaster* (51.9 kDa) based on the *Drosophila pah* gene cDNA sequence (Morales *et al.*, 1990). Negative controls using ascites fluid, not containing anti-PAH antibodies, or total mouse IgG showed no band reacting at this position, which confirms the specificity of the detection system. This band cannot be the tyrosine hydroxylase protein, since the molecular mass of the latter (58 kDa) (Neckameyer & Quinn, 1989) is large enough to allow its discrimination from 51 kDa proteins in our electrophoresis system.

The developmental profile of the putative PAH protein (Fig. 2) shows a strong band of CRM at the beginning of pupariation and a smaller one at the end of the pupal stage (pharate adult). The amount of protein detected in pupae is higher than in third instar larvae, and the latter is higher than that in adult flies. Immediately after the emergence of the adult the amount of protein decreases, being difficult to detect beyond the 4th day. These visually estimated amounts of protein correlate relatively well with PAH enzyme activities during development (Bel *et al.*, 1990; Bel *et al.*, 1992). Quantitative differences between the two detection systems could reflect different levels of active and inactive protein, since it is known that mammalian PAH and other aromatic amino acid hydroxylases are subject to post-translational regulation (Kaufman, 1990).

The major role of PAH in *Drosophila* seems to be the production of a sufficient amount of tyrosine for cuticle formation and hardening. In that sense, the peak at pupariation would be correlated with the hardening of the larval cuticle, and the levels of protein during pupation and at the pharate adult formation with the synthesis of the adult cuticle.

Additional evidence that this CRM band corresponds to the PAH protein is the tissue distribution. Morales *et al.* (1990) have shown that at the larval stage the gene for PAH is only expressed in the fat body. We have analysed the tissue distribution of the putative PAH protein and found that, at this stage of development, only the fat body shows detectable amounts of protein, with no protein in the salivary glands, brain or haemolymph. In young adults, on the other hand, PAH enzyme activity is mainly restricted to the head (Bel *et al.*, 1992) which correlates very well with the amounts of PAH protein detected in Western blots (Fig. 2).

Final evidence that this CRM is the PAH protein was provided by the analysis of two *Henna* mutants. The virtual absence of the CRM band in *Hn^{r3}* and its decreased amount in *Hn^r* (Fig. 4) correlates with the PAH activity in these mutants (Table 1). The identity of *Henna* as the structural gene for PAH requires confirmation by molecular and biochemical analyses of several *Henna* mutant alleles. The confirmation of this finding will have important implications for PAH enzyme functions, since *Henna* mutant strains are defective not only in aromatic amino acid metabolism (Bel & Ferré, 1989) but also in pteridine biosynthesis (Ferré *et al.*, 1986; Guillamón & Ferré, 1988; Escriche & Silva, 1992). This proposed function of PAH may not only be specific to insects, since the function of PAH as a tetrahydropterin oxidase *in vitro*, without hydroxylation of the aromatic amino acid substrate, has been shown for the mammalian enzyme (Fisher & Kaufman, 1973; Davis & Kaufman, 1989). On the other hand, it has recently been reported that oxidation of the naturally occurring 7-tetrahydrobiopterin by purified rat liver PAH is 85 % uncoupled (Davis & Kaufman, 1991). The fact that this pterin is excreted in relatively high amounts by hyperphenylalaninaemic patients (Curtius *et al.*, 1988) allows us to hypothesize that PAH has a tetrahydropterin oxidase function under physiological conditions.

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