A novel two-dimensional polyacrylamide-gel pattern, which may be due to allelic genes, of phenylalanine hydroxylase in monkeys

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Two-dimensional gel electrophoresis of immunopurified monkey liver phenylalanine hydroxylase showed a novel form of the enzyme, in 4 out of 24 monkeys, in which each polypeptide spot was split into a doublet with the same charge but slightly different mobility in the sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis (as opposed to the isoelectric-focusing) dimension. Phenylalanine hydroxylase formed by translation of RNA from a liver containing the novel form showed the doublet pattern, suggesting that it is due to differences in mRNA. By analogy with the rat, this mRNA difference could be due to allelic genes.

INTRODUCTION

Mammalian liver phenylalanine hydroxylase (phenylalanine 4-mono-oxygenase, EC 1.14.16.1) catalyses the conversion of phenylalanine into tyrosine and is principally found in the liver [for reviews, see Cotton (1977), Kaufman (1976) and Goodwin (1979)]. Two-dimensional polyacrylamide-gel analysis of purified monkey liver phenylalanine hydroxylase has shown that the enzyme consists of two different M_r forms, designated H (apparent M_r 50 000) and L (apparent M_r 49 000), each containing three isoelectric forms (Choo *et al.*, 1979; Cotton *et al.*, 1980). Recently we demonstrated that, in human and monkey livers, the two M_r forms (H and L) represent the phosphorylated and dephosphorylated form of phenylalanine hydroxylase respectively (Smith *et al.*, 1984). In the rat, however, we have previously shown that the two apparent- M_r forms of rat liver phenylalanine hydroxylase are isoenzymes encoded by different mRNA species (Mercer *et al.*, 1983) deriving from allelic genes (Mercer *et al.*, 1984). In the present paper we report the discovery of a novel form of phenylalanine hydroxylase, present in some monkey livers, in which each spot seen on two-dimensional gels of the 'standard' pattern is split into two of differing mobility in the 'SDS' dimension. Comparison of translation products of RNA from 'novel' and 'standard' monkey liver shows a similar doublet pattern in the former, suggesting the novel pattern results from translation of different mRNA species and, by analogy with the rat, is possibly due to allelic phenylalanine hydroxylase genes in the monkey.

MATERIALS AND METHODS

Monkey livers, excised under anaesthesia and immediately snap-frozen in slices on solid CO₂, were kindly donated by Commonwealth Serum Laboratories, Parkville, Vic., Australia, and were derived from wild monkeys (*Macaca irus*) from Malaysia. Phenylalanine hydroxylase was purified from monkey liver extracts by affinity chromatography using a monoclonal antibody (PH1-1) immuno-affinity column essentially as described by Choo

et al. (1981). The phenylalanine hydroxylase preparations were 95% homogeneous by densitometry of Coomassie Brilliant Blue-stained SDS/polyacrylamide gels.

Preparation of total RNA from monkey livers was carried out by using the method of Chirgwin *et al.* (1979) with guanidinium chloride in place of guanidinium thiocyanate (Wake & Mercer, 1985). The RNA was translated in a rabbit reticulocyte lysate system exactly as described by Mercer *et al.* (1983), the nascent polypeptides were labelled with [³⁵S]cysteine (400 µCi/ml of incubation mixture) and the total RNA concentration was 200 µg/ml. The final mixture (100 µl) was incubated for 80 min at 30 °C and the phenylalanine hydroxylase isolated by immunoprecipitation with a rabbit anti-(rat phenylalanine hydroxylase) antibody as described previously (Mercer *et al.*, 1983).

Two-dimensional gel electrophoresis was carried out by the procedure of O'Farrell (1975), as modified by Choo *et al.* (1981). Isoelectric focusing was performed at room temperature for 16 h at 400 V, followed by a further 800 V for 1 h. The second-dimension gels were SDS/9% (w/v) polyacrylamide (Laemmli, 1970) and electrophoresis was a constant current of 25 mA for 5–6 h. Fixation, staining and fluorography were as described previously (Mercer *et al.*, 1983).

RESULTS AND DISCUSSION

Monkey liver phenylalanine hydroxylase was purified from crude extracts by using a monoclonal antibody column (Choo *et al.*, 1981). The protein was examined by two-dimensional gel electrophoresis and, from most (20) of the monkeys, the 'standard' pattern consisting of two M_r classes of polypeptides, H (phosphorylated) and L (dephosphorylated), each containing three polypeptides of differing charges, was observed. This pattern is shown in Fig. 1(a) and is essentially the same as reported by Smith *et al.* (1984). Another four monkey livers, however, yielded phenylalanine hydroxylase in which each spot in the standard pattern was present as a doublet of identical charge but slightly different mobility on SDS/polyacrylamide-gel electrophoresis (Fig. 1b). In the discussion

Abbreviations used: H-form and L-form, enzyme forms with higher and lower M_r respectively; SDS, sodium dodecyl sulphate.

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Fig. 1. Two-dimensional gel analysis of phenylalanine hydroxylase immunoaffinity-purified or produced by cell-free translation of monkey liver RNA

(a, b) Phenylalanine hydroxylase was isolated from monkey livers by using a monoclonal antibody column (Choo *et al.*, 1981) and analysed by two-dimensional gel electrophoresis as described in the Materials and methods section. The protein was detected by Coomassie Brilliant Blue staining. Only the portion of the gel containing phenylalanine hydroxylase polypeptides is shown. The left side of the gel represents the basic end of the isoelectric-focusing gel where the protein was loaded. The arrows indicate the positions of the different apparent- M_r classes (H and L). The monomers forming the doublet in the novel pattern are indicated by α and β respectively. (a) The standard pattern of phenylalanine hydroxylase; (b) the novel pattern of phenylalanine hydroxylase. (c, d). Total RNA from monkey livers was prepared as described in the Materials and methods section and translated in a rabbit reticulocyte-lysate system with [35 S]cysteine. After addition of 5 μ g of monkey phenylalanine hydroxylase as a carrier, immunoprecipitation was carried out with a phenylalanine hydroxylase-specific serum (Mercer *et al.*, 1983). The immunoprecipitate (3×10^4 c.p.m.) was subjected to two-dimensional gel electrophoresis and fluorography (Bonner & Laskey, 1974). Only the portions of gel containing the phenylalanine hydroxylase polypeptides are shown. (c) Immunoprecipitated translation products of RNA from a standard monkey liver. (d) Translation products of RNA from a 'novel' monkey liver.

below we refer to the apparent higher- M_r form of the doublet as ' α ' and the lower as ' β '; thus a higher spot in the L-class is ' $L\alpha$ ' and the lower is ' $L\beta$ '.

We consider that the β -form is not a simple proteolysis product of the α -form, since repeated preparations of the enzyme from the same liver always gave the same pattern, and all the preparations from different livers had very similar ratios of α to β (about 2:3 as estimated by densitometry); this ratio might have been expected to vary if the β -form were a proteolysis product of the α -form. In addition, isolation of the protein in the presence of the serine-proteinase inhibitor phenylmethanesulphonyl fluoride did not change the relative proportions of α and β . The tetrahydrobiopterin-dependent phenylalanine hydroxylase activity in these livers, estimated by the method of Choo *et al.* (1979), was identical with that in

livers giving the standard pattern (results not shown); if proteolysis had been extensive, the novel pattern preparations might have been expected to have a lower specific activity.

The consistency of the α/β ratio suggested, by analogy with the allelic genes of the rat (Mercer *et al.*, 1984), that the novel pattern could come from monkeys that are heterozygotes for two phenylalanine hydroxylase alleles, one coding for the α -form and the other for the β -form. To further investigate this possibility, we isolated RNA from a novel- and a standard-pattern monkey liver and translated these in a rabbit reticulocyte cell-free system. The phenylalanine hydroxylase formed *in vitro* was labelled by incorporation of [35 S]cysteine and immunoprecipitated with a phenylalanine hydroxylase-specific antiserum. Analysis of the immunoprecipitate by two-

dimensional gel electrophoresis (Fig. 1c) showed that RNA from the standard liver translated to give a single L-row. A trace of H-row (the phosphorylated form) is just detectable on the original fluorogram. The different isoelectric forms were not well resolved in this case, hence the pattern appears as a smear. This smearing is occasionally observed with the purified protein. Apart from the position on the gels, the identity of the immunoprecipitated material was confirmed by failure to precipitate with control serum and competition with purified phenylalanine hydroxylase (results not shown). The low amount of phosphorylated species (H-forms) was presumably due to a low level of protein kinase activity in the reticulocyte lysate (Montarras *et al.*, 1981). RNA from a 'novel' monkey, in contrast, yielded phenylalanine hydroxylase that showed a doublet-spot pattern, presumably corresponding to α and β . Slightly more of the lower- M_r (β -form) was formed.

The evidence presented here shows that a subpopulation of monkeys has an unusual form of phenylalanine hydroxylase in which each spot is split into a doublet. Furthermore, the translation results suggest that the α - and β -forms are encoded by separate mRNA species and these two mRNA species may be coded for by allelic genes. The evidence for this, in addition to the mRNA data, is that the forms have the same charge and are present in a consistent ratio. Both these properties are shared by rat heterozygotes for the W- and L-alleles of phenylalanine hydroxylase (Mercer *et al.*, 1984), except that equal amounts of the two allelic proteins are usually present in the rat heterozygote, whereas in the monkey the β -form seems to be in slight excess. Since this excess of β -form is also seen in the translation products, this would suggest that if two putative allelic genes do exist in the monkey, then they may differ in their rate of transcription, the stability or translational efficiency of their mRNA products.

Formal proof of allelic genes would require controlled breeding experiments as we described for our rat colony (Mercer *et al.*, 1984), but this is quite impractical with the monkeys available to us, since they are captured wild and

not kept for breeding. From our evidence, the two alleles for phenylalanine hydroxylase appear to be not only restricted to laboratory rats, but are also found in monkeys, and presumably in man. We have preliminary data which suggests that wild rats and mice have the two alleles as well as have some cats, so perhaps the phenomenon will be found in more mammals.

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