α-HYDROXYACID OXIDASE GENETICS IN THE MOUSE: EVIDENCE FOR TWO GENETIC LOCI AND A TETRAMERIC SUBUNIT STRUCTURE FOR THE LIVER ISOZYME*

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ABSTRACT

We have examined a polymorphism for liver GOX in inbred strains of the mouse *Mus musculus*. Genetic studies demonstrated that the two phenotypes for this enzyme present in BALB/C and NZC parental strains segregated as though they were controlled by codominant alleles at a single autosomal locus (GOX) which mapped closely to the agouti locus in linkage group V. Kidney HAOX activity is invariant in these inbred strains and is encoded by a separate genetic locus designated HAOX. BALB/C × NZC F_1 hybrid mice exhibited three intermediate forms of liver GOX activity, in addition to the parental enzymes, which is consistent with a tetrameric subunit structure.

THE presence of a flavoprotein enzyme in peroxisomes (microbodies) and the cytoplasm of mammalian tissues which catalyzes the oxidation of α-hydroxyacids by O_2 is well documented (LEIGHTON *et al.* 1968; SHNITKA and TALIBI 1971). The enzyme, α-hydroxyacid oxidase (E.C.1.1.3.1.) catalyzes the general reaction:

$$\text{R-CHOH-COOH} + O_2 \longrightarrow \text{R-CO-COOH} + H_2O_2$$

Previous studies have demonstrated that the enzyme exists in mammalian tissues as multiple molecular forms (isozymes) and oxidizes a variety of α-hydroxyacids (ROBINSON *et al.* 1962; ALLEN and BEARD 1965; DOMENECH and BLANCO 1967). Rat and pig liver α-hydroxyacid oxidases preferentially oxidize short-chain aliphatic hydroxyacids, exhibiting maximal activity with glycollate, and are named glycollate oxidases (GOX) (NAKANO *et al.* 1968; SCHUMAN and MASSEY 1971). The rat kidney enzyme oxidizes long-chain aliphatic hydroxyacids most efficiently and also uses L-amino acids as substrates. This enzyme is referred to as L-amino acid oxidase (LAOX; E.C.1.4.3.2) or α-hydroxyacid oxidase (HAOX) (BLANCHARD *et al.* 1944; NAKANO and DANOWSKI 1966; SALVATORE, ZAPPIA and CORTESE 1966). Alternatively, pig kidney contains both short and long-chain α-hydroxyacid oxidases (ROBINSON *et al.* 1962). Thus it appears from these studies that two major isozymes of α-hydroxyacid oxidase activity exist in these tissues. These are designated in this communication as GOX and HAOX,

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which refer to the short-chain and long-chain α-hydroxyacid oxidases, respectively.

We report in this paper the electrophoretic behavior of GOX and HAOX activity from liver and kidney extracts of inbred strains of mice. A polymorphism for liver GOX was observed and subsequently used in genetic studies on this enzyme. The results demonstrate that the liver and kidney isozymes are encoded by separate genetic loci, with the GOX locus being located on linkage group V of the mouse. They also indicate that liver GOX has a tetrameric subunit structure.

MATERIALS AND METHODS

Mouse strains: Two inbred strains of *Mus musculus*, NZC and BALB/C, were routinely used for the studies. Matings between male BALB/C mice and female NZC mice were made and the F_1 offspring were backcrossed with NZC mice to examine segregation and the possible linkage relationships of the GOX locus to coat color genetic loci. Other mouse inbred strains analyzed included C_s^A, C_s^B, C_s^D, CBA/H and C57BL/Ka Lw.

Homogenate preparation: Livers and kidneys were excised from mice freshly killed by exsanguination, rinsed in cold distilled water and homogenized in 10% sucrose/10 mM phosphate buffer, pH 7.4 (at dilutions as indicated in Figure 1). The homogenates were then centrifuged (100,000 g, 45 minutes) prior to electrophoresis.

Gel electrophoresis and histochemical staining: Homogenate supernatants were subjected to zone electrophoresis on horizontal 10% starch gels at 4° with Tris-glycine buffer (20 mM glycine), pH 9.0. A voltage gradient of 20 V/cm was applied for 16 hours. The gels were then sliced, stained for activity and photographed.

The principle of the staining technique depends upon the coupling of hydrogen peroxide production by the oxidase to the reduction of a chromagen by horseradish peroxidase (Robinson and Lee 1967). After slicing, the starch gels were covered by 25 ml 1% agar overlay containing 900 μmoles Tris-HCl buffer (pH 7.4), 200 μmoles α-hydroxyisocaproic acid (a common substrate for GOX and HAOX), 3 mgm o-dianisidine dihydrochloride and 100 units of horseradish peroxidase (Sigma Type II), then incubated at 37° until bands of intensity suitable for photographing were obtained. This staining method is modified from that used by Lewis and Harris (1967) for peptidase staining.

RESULTS AND DISCUSSION

Following electrophoresis, the mouse liver extract exhibited a major form of activity together with several anodally migrating secondary bands or "subbands". Liver GOX activity from NZC mice migrated more slowly to the anode than the BALB/C enzyme. GOX activity from the NZC × BALB/C hybrid exhibited three intermediate forms of activity in addition to the parental forms (Figure 1). The occurrence of this five-isozyme pattern for a protein encoded by a heterogeneous locus is consistent with a tetrameric subunit structure for the enzyme: GOX -A_4, A_3A', $A_2A'_2$, AA'_3 and A'_4 (Shaw 1965).

The GOX "subbands" behaved identically to the major enzyme in these and subsequent genetic studies involving F_2 individuals. This result is similar to those reported for the mitochrondrial isozyme of malate dehydrogenase (MDH-B_2) (Shows, Chapman and Ruddle 1970), mouse muscle lactate dehydrogenase (LDH-A_4) (Dudman 1969), and for mouse liver catalase (Holmes 1972). For these enzymes, epigenetically modified forms contribute to the multiplicity observed on starch gel zymograms. The nature of this modification is known for

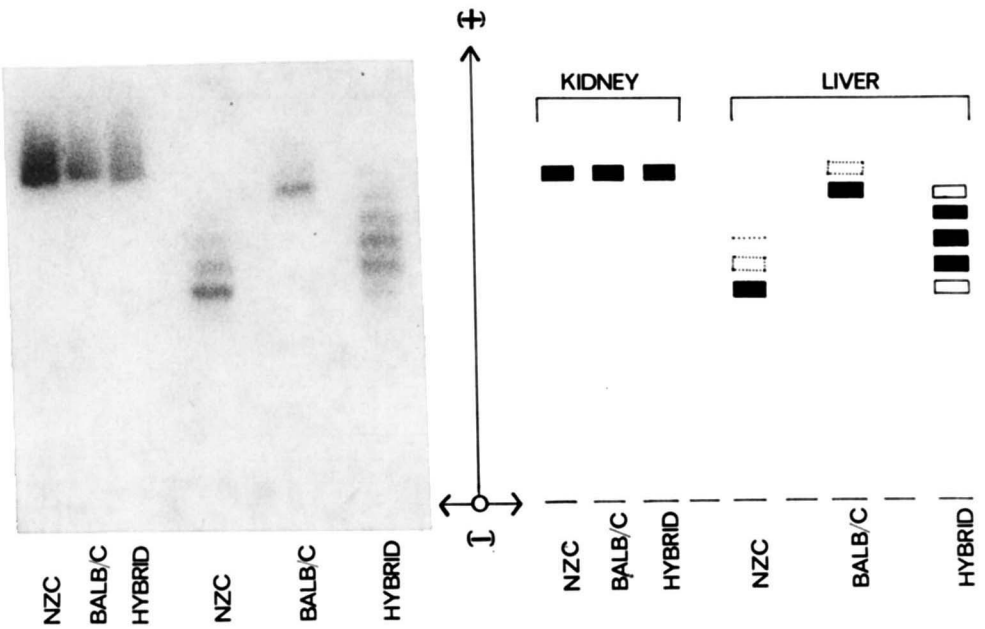

FIGURE 1.—Starch gel zymogram of supernatant α-hydroxyacid oxidase isozymes of mouse inbred strains. Homogenate concentrations: BALB/C kidney—33% w/v; NZC and F_1 BALB/C × NZC hybrid kidney—25% w/v; NZC liver—33% w/v; BALB/C and F_1 BALB/C × NZC hybrid liver—50% w/v. See text for details.

mouse LDH-A_4 with glutathione binding to the free sulfydryl groups on the enzyme producing electrophoretic heterogeneity. Other epigenetic modifications may involve acetylation, deamidation, sialic acid binding, binding of other low molecular weight acidic molecules, or the oxidation of sulfydryl groups.

HAOX activity of mouse kidney extracts migrated as a single form electrophoretically distinct to the GOX activity from liver extracts (Figure 1). The electrophoretic migration of HAOX activity was identical for both NZC and BALB/C mice, suggesting that these enzymes are identical in both strains. These results are in contrast to the variation observed for liver GOX activity and strongly indicate that GOX and HAOX are encoded by separate genetic loci. Five other strains of mice, C_s^A, C_s^B, C_s^D, CBA/H and C57BL/KaL, were examined and shown to be phenotypically identical to BALB/C mice with respect to GOX and HAOX electrophoretic migration.

NZC × BALB/C F_1 hybrids when backcrossed to NZC mice produced 40 mice displaying the 5-banded hybrid pattern and 35 mice with the NZC-phenotype pattern. The ratio of homozygotes to heterozygotes does not differ significantly from the expected 1:1 for the locus (GOX-A:$\chi^2 = 0.33$, 1 d.f.). A uniform expression of the GOX phenotype was observed among both male and female prog-

TABLE 1

Distribution of GOX-A and A (agouti) phenotypes among backcross progeny from (NZC × BALB/C) F_1 and NZC

	aa	Aa	Total
GOX-A′ GOX-A′	33	2	35
GOX-A′ GOX-A	2	38	40
Total	35	40	75

eny regardless of the sex and the hybrid parent, indicating that GOX-A is controlled by an autosomal locus.

The rat liver GOX and rat kidney HAOX enzymes have been purified and a molecular weight of 300,000 reported (NAKANO *et al.* 1968; NAKANO, TARUTANI and DANOWSKI 1968). This molecular weight is sufficiently large to contain four subunits, as is indicated from our results. USHIJIMA and NAKANO (1969) have reported the FMN content of rat liver GOX as 2 moles FMN per mole of enzyme—which would further indicate, assuming a tetrameric subunit structure, that one mole of FMN is present for every two subunits. This is in contrast to results obtained for crystalline spinach glycollate oxidase (FRIGERIO and HARBURY 1958). For this enzyme, ultracentrifugal analyses and flavin determinations indicated a tetrameric subunit structure with one molecule of FMN being bound per monomer.

The two inbred parental strains, NZC, of coat color phenotype brown non-agouti (genotype CCaabb) and BALB/C, of albino coat color (genotype ccAAbb), gave an F_1 hybrid with brown agouti coat color (CcAabb). When the F_1 hybrids were backcrossed with NZC mice, the GOX-A locus segregated with the agouti locus, except in 4 of the 75 progeny (Table 1).

Distribution of all four classes differed significantly from the 1:1:1:1 ratio expected for unlinked loci ($\chi^2 = 60.5$, 3 d.f.), suggesting that the GOX-A and agouti loci are linked, with a recombination frequency of 5.3%.

It has been demonstrated that the agouti locus is in linkage group V of mouse (GREEN 1966). Thus the GOX-A locus can be mapped in this group. Since no variant has yet been observed for HAOX, it was not possible to ascertain the linkage relationship between HAOX- and GOX-encoding loci.

The inbred strains CBA/H, NZC, BALB/C and C57BL/Ka Lw were originally donated by the Walter and Eliza Hall Institute, Melbourne. The C_8^A, C_8^B, and C_8^D inbred strains were kindly donated by DR. ROBERT N. FEINSTEIN of the Argonne National Laboratory, Illinois.

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