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Biochemistry

Peroxisomes in wild-type and rosy mutant *Drosophila melanogaster*

(catalase/xanthine oxidase/xanthine dehydrogenase/inherited metabolic disease/Malpighian tubule)

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ABSTRACT **This study shows that peroxisomes are abundant in the Malpighian tubule and gut of wild-type Oregon R *Drosophila melanogaster* and that the peroxisomal population of the rosy-506 eye-color mutant differs from that of the wild type. Catalase activity in wild-type flies is demonstrable in bodies of appearance and centrifugal behavior comparable to the perixosomes of vertebrate tissues. Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.22) activity of the Malpighian tubule of wild-type flies is demonstrable cytochemically in bodies like those containing catalase. The rosy-506 mutant flies, with a deletion in the structural gene for xanthine dehydrogenase (xanthine:NAD$^+$ oxidoreductase, EC 1.1.1.204), lack cytochemically demonstrable peroxisomal xanthine oxidase activity. In addition, perixosomes in the rosy-506 mutants show less intense cytochemical staining for catalase than those in wild-type flies, and biochemical assays indicate that catalase in the rosy mutant is much more accessible to substrate in the absence of detergent than in the wild type. Thus, the rosy-506 mutation appears to affect peroxisomes and may mimic aspects of the defects of peroxisomes in some human metabolic disorders.**

Deficits in one or more of the peroxisomal enzymes and possible defects in peroxisomal structure are correlated with certain human inherited metabolic diseases (1–4). These diseases are still poorly understood, in part because different tissues show different effects of the mutation and in part because alterations in single genes can seemingly have multiple effects on the peroxisomes. Studies of the disorders are made difficult by their rareness and by the obvious limits of working with human material. It would be helpful to study analogues of the disorders in animals amenable to detailed analysis, but thus far such analogues have not been available.

We chose to initiate our explorations for peroxisomal mutants in *Drosophila* by comparing wild-type Oregon R strain flies with the rosy-506 eye-color mutant, in which 90% of the structural gene for xanthine dehydrogenase (xanthine:NAD$^+$ oxidoreductase, EC 1.1.1.204) is deleted (5, 6). This protein is a bifunctional oxidoreductase that can act as a dehydrogenase or as an oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.22) depending upon substrate and acceptor availability (7, 8). The oxidase activity of the enzyme is critical to the degradation of purines in many organisms; the dehydrogenase function is central to the formation of pterine-based eye-color pigments in *Drosophila*. We suspected that this protein might be peroxisomal because (*i*) when acting as an oxidase, it produces hydrogen peroxide, a common feature of peroxisomal oxidases; (*ii*) it requires flavin in generating peroxide, as is found for many other peroxisomal oxidases; and (*iii*) its role in purine degradation is potentially functionally linked to activity of allantoicase and uricase, known peroxisomal enzymes (9). The subcellular localization of xanthine oxidase is not clearly established. Some cell fractionation studies show most of the enzyme to be soluble (9, 10), while other investigations report some xanthine oxidase cosedimenting with peroxisomal enzymes (11, 12).

The study reported here identifies peroxisomes in the Malpighian tubule and gut of adult *Drosophila* wild-type Oregon R and rosy-506 strains, by virtue of their content of catalase, the peroxisomal "marker" enzyme. To localize xanthine oxidase, we have adapted cytochemical methods used to demonstrate other oxidases. With these methods, we have shown the presence of xanthine oxidase in peroxisomes of wild-type flies and the absence of this enzyme in rosy-506 flies. We also find signs that, as in the human mutations, a relatively simple genetic change can have complex effects on the peroxisomes. Preliminary reports of our findings have been published (13, 14).

MATERIALS AND METHODS

Drosophila melanogaster of the Oregon R wild-type strain and rosy-506 eye-color mutant (containing a structural gene deletion in the xanthine dehydrogenase locus on an Oregon R background) were grown on standard cornmeal/agar medium at room temperature. Adult flies of both sexes were quick-frozen on dry ice; abdominal organs were dissected either into 0.25 M sucrose/1% albumin to protect catalase activity for biochemical study (15) or into aldehyde fixative (see below) for microscopic study. Reproductive organs, eggs, and fat were removed as completely as possible and discarded. It was sometimes feasible to separate the gut and Malpighian tubule before embedding the tissues for microscopy. Generally, however, the two organs were handled together.

Microscopy. Fixation was carried out for 30–60 min at 4°C in either 2% glutaraldehyde/1% paraformaldehyde (freshly prepared)/0.1 M sodium cacodylate buffer, pH 7.4 (16), or in 0.5% glutaraldehyde/0.1 M Pipes, pH 7.4/2% sucrose (17).

Cytochemistry. Catalase was demonstrated cytochemically by an alkaline 3,3′-diaminobenzidine (DAB) technique (18). Tissue fixed in either fixative was preincubated for 30–60 min in medium lacking hydrogen peroxide and then incubated in complete medium for 60 min at room temperature and 60 min at 37°C or 60–120 min at 37°C. The medium was replaced every 30 min. As controls, some sections were incubated in substrate-free medium, and other sections were incubated in medium containing 0.1 M 3-amino-1,2,4-triazole, a noncompetitive inhibitor of catalase (19).

Xanthine oxidase was demonstrated in tissue fixed in the Pipes-buffered fixative by a modification of the cerium perhydrate method developed for work on peroxisomes (17, 20–24). A saturated solution of xanthine (approximately 2 mM) in 0.1 M Pipes (pH 7.6–7.8) served as substrate; FAD and NAD, each at 10 mM (10), 0.0001–0.0002% Triton X-100 (17), 0.005 M $CeCl_3$, and 0.1 M 3-amino-1,2,4-triazole were added to this solution. Preincubation in substrate-free medi-

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Abbreviation: DAB, 3,3′-diaminobenzidine.

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um for 30–60 min at room temperature was followed by incubation in complete medium for 90 min at room temperature followed by 90 min at 37°C. Medium was replaced every 30 min. Control sections either were preincubated and incubated in substrate-free medium or were preincubated in substrate-free medium from which 0.1 M 3-amino-1,2,4-triazole had also been omitted and then were incubated in this same mixture containing substrate (21).

Unincubated tissue and incubated tissue were postfixed for 1 hr in 1–2% osmium tetroxide in water, dehydrated by passing through ethanol and propylene oxide, and embedded in epoxy resin (LX-112, Ladd Research Industries, Burlington, VT). "Thick sections" (250 nm) were used to judge overall morphological integrity and to locate the regions of Malpighian tubule and gut of interest. "Thin sections" (60–70 nm) were examined in a Philips 201 electron microscope operating at 60–80 kV.

Biochemistry. Abdominal organs from 10 flies per 0.5 ml of sucrose/albumin medium were hand-homogenized by using six up/down strokes of a glass/Teflon Potter–Elvejehm-type homogenizer. Catalase activity was measured either in the whole homogenates or after the homogenates had been separated into subcellular fractions. These fractions were obtained by standard differential centrifugation methods using a Sorvall RC-5 centrifuge equipped with an SS-34 rotor (15, 25). Fraction I was sedimented at 2700 × *g*·min; fraction II at 450,000 × *g*·min; fraction III was the supernatant from the second sedimentation step. The postnuclear supernatant (i.e., the supernatant after sedimentation of fraction I) was subjected to density gradient sedimentation in a sucrose/water gradient with density limits of 1.12–1.26 using a Beckman model L-5 centrifuge equipped with a Ti50 rotor (15). Catalase activity was determined spectrophotometrically with a Beckman DU spectrophotometer by measuring the disappearance of hydrogen peroxide either directly at 240 nm or indirectly as a titanium persulfate complex absorbing at 510 nm (15).

RESULTS

Cytochemistry. Catalase-positive, DAB-stained peroxisomes were readily demonstrable in epithelial cells of Malpighian tubules in wild-type Oregon R strain flies (Fig. 1). The cytochemical reaction was not seen when tissue was incubated in the presence of 3-amino-1,2,4-triazole or in medium lacking hydrogen peroxide. The DAB-stained structures were enclosed by a single membrane, were 0.2–0.5 μm in cross-sectional diameter, and usually were round to oblong in shape but occasionally appeared as multiple, interconnected bodies. The larger bodies had dense regions in their matrix and thus resemble somewhat the vertebrate hepatic or renal peroxisomes. No preferential intracellular location of the organelle was noted.

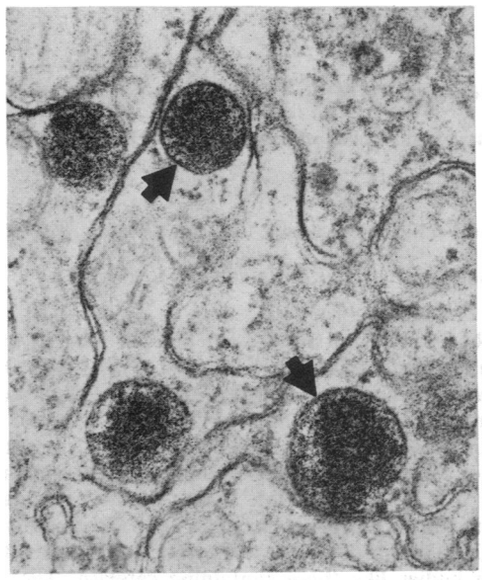

FIG. 2. Xanthine oxidase activity, visualized as cerium deposits, is found in perixosomes (arrows) of the Malpighian tubule of wild-type *Drosophila*. (×40,000.)

Cerium deposits due to xanthine oxidase activity were found in the Malpighian tubules of wild-type flies within membrane-delimited bodies similar to the catalase-containing bodies in appearance and number (Fig. 2). No reaction product was seen in similar bodies in substrate-free controls or in tissue of the rosy-506 mutant (Fig. 3). The reaction was absent from wild-type Malpighian tubule after incubation in the xanthine oxidase medium from which 3-amino-1,2,4-triazole was omitted, suggesting that the xanthine oxidase activity is in the same structures as catalase (21).

In rosy material incubated to demonstrate catalase, we had the initial impression that reactive peroxisomes were rare in Malpighian tubule. Wild-type tissue proved readily distinguishable from rosy tissue in systematic blind comparisons of

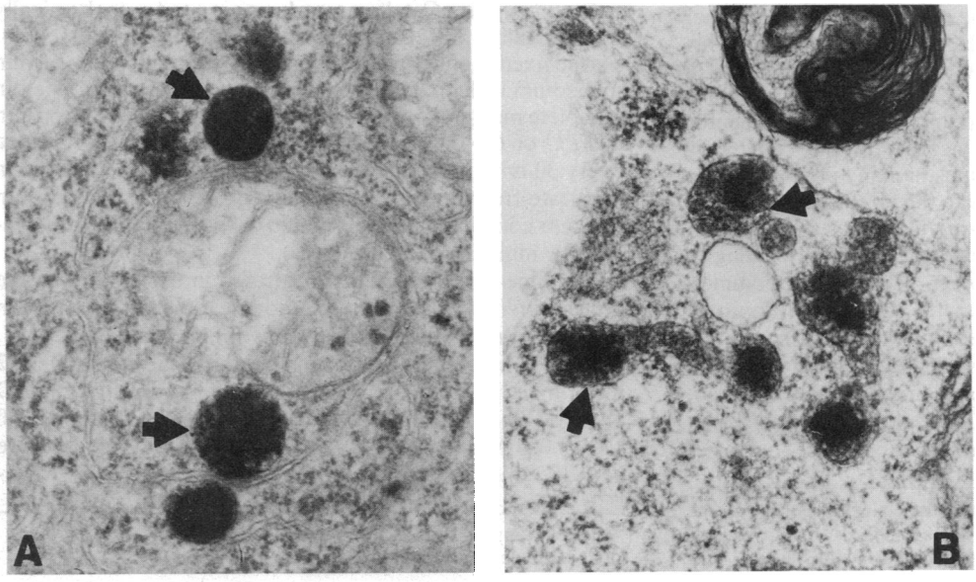

FIG. 1. (*A*) Catalase activity visualized as the DAB reaction product is localized to strongly stained peroxisomes (arrows) of Malpighian tubule of wild-type *Drosophila*. (×40,000.) (*B*) A focal density is evident in the matrix of lightly DAB-stained peroxisomes. (×37,500.)

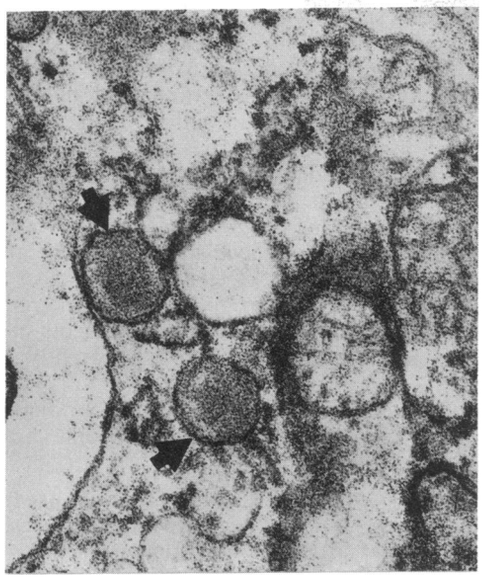

FIG. 3. Peroxisomes (arrows) of the Malpighian tubule of rosy-506 mutant flies fail to show cerium deposits after incubation for xanthine oxidase activity. Compare with Fig. 2. (×38,500.)

catalase-incubated material on the basis of the scarcity of obviously reactive bodies in the rosy material. Upon careful examination, however, we found that rosy-506 tissues did have reactive peroxisomes but that the contrast of these bodies against the cytoplasm was much less strong than in wild-type tissue (Fig. 4). Counts of 200 DAB-reacted bodies, taken from 80 randomly selected electron microscopic fields, showed 2–3 times as many markedly reactive peroxisomes per unit area of cytoplasm in wild-type Malpighian tubule as in the rosy-506 material. We could not tell whether the total numbers of peroxisomes in fact differed between rosy and wild type because many profiles of *Drosophila* peroxisomes lacked a sufficiently distinctive morphology to be distinguished unequivocally from other organelles when the cytochemical reaction product was absent.

Catalase-containing peroxisomes were readily demonstra-

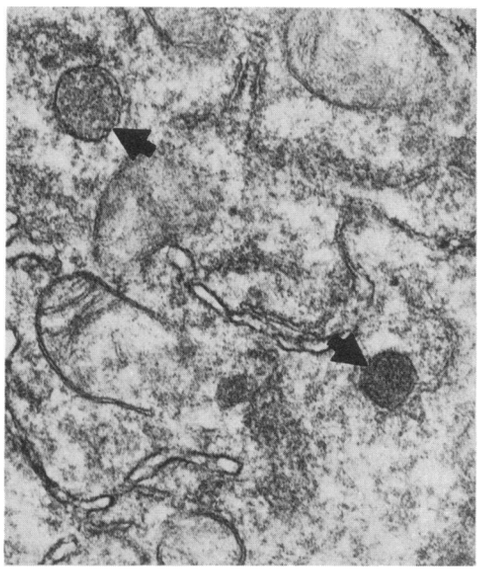

FIG. 4. Peroxisomes (arrows) of the Malpighian tubule of rosy-506 flies show low levels of catalase reaction after incubation with DAB. Compare with Fig. 1. (×37,000.)

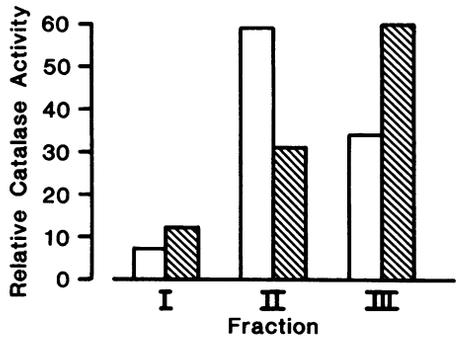

FIG. 5. Catalase activity after differential sedimentation is predominantly recovered in fraction II, the organellar fraction, in wild-type flies (□) and in fraction III, the soluble fraction, in rosy flies (▨). The assays were performed in the presence of 0.1% Triton X-100. Relative activity is the percentage of the total activity of the loaded material recovered in each fraction (n = 5).

ble in the gut epithelium of wild-type flies, and some such bodies were also seen in rosy flies. The bodies in gut were somewhat smaller than those in the Malpighian tubule, resembling in size the microperoxisomes of vertebrates (18). No xanthine oxidase was found in peroxisomes in gut in either the wild-type or the rosy-506 flies.

Biochemistry. In preliminary work we used a fluorescence assay method to verify the absence of detectable xanthine oxidase activity in homogenates and subcellular fractions of rosy-506 mutant flies and the presence of this enzyme in wild-type flies (26). In our hands this technique was not sufficiently sensitive for detailed quantitative work on cellular fractions obtainable from the small samples of tissue that realistically can be processed from adult *Drosophila*. Therefore, we focused our attention on catalase activity.

Overall levels of catalase activity were similar in wild-type and rosy flies, yet the enzyme showed marked differences in behavior. In differential centrifugation studies, catalase activity was predominantly sedimentable in the wild-type flies and predominantly soluble in the rosy mutant flies (Fig. 5). After density gradient centrifugation of the postnuclear supernatant of a homogenate of wild-type flies, catalase activity showed the expected distribution, with sedimentable activity appearing at a mean density of 1.21–1.22 and soluble activity appearing at the load position (Fig. 6; see refs. 9, 15, and 25). We did not pursue density gradient sedimentation of

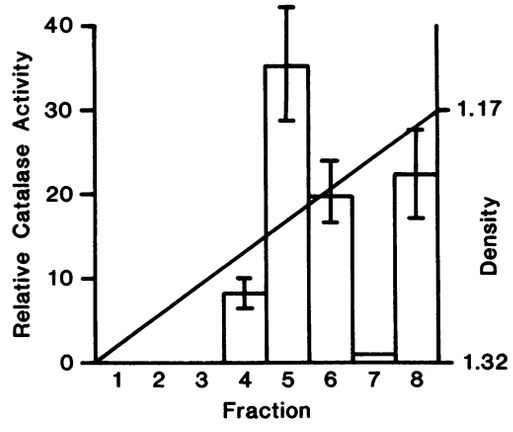

FIG. 6. Sucrose density gradient distribution of catalase activity in wild-type tissues, measured in the presence of 0.1% Triton X-100. Enzyme activity is notable at a mean sucrose density of 1.21–1.22, similar to that of vertebrate peroxisomes, and in the load fraction, corresponding to "soluble" material (n = 3).

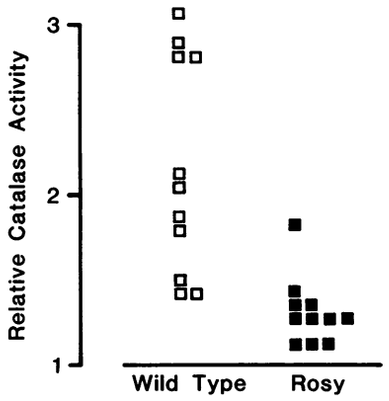

FIG. 7. Relative catalase activity here is the ratio of total activity of the postnuclear supernatant (measured in the presence of 0.1% Triton X-100) to free activity (measured in the absence of detergent). The lower ratio found for the rosy flies indicates less structural latency than in wild-type flies. Each point represents 1 of 11 separate measurements.

equivalent material from the rosy mutant because in pilot work the catalase activity was spread throughout the lighter regions of the gradient as would be expected from its soluble distribution in the differential centrifugations.

Whether measured in unseparated homogenates, in postnuclear supernatants, or in the gradient fractions, the catalase of wild-type flies exhibited substantial "latency" as expected for a particle-bound enzyme (9, 15, 25). That is, Triton X-100 detergent treatment resulted in a 2-fold increase in demonstrable enzyme in the wild-type flies. The detergent had much less effect on the preparations from the rosy-506 flies (Fig. 7).

DISCUSSION

Much less is known about perixosomes of invertebrates than of vertebrates. Among insects, peroxisomes have been identified cytochemically in Calpodes, Gryllus, and Photuris, where peroxisomes were studied in larval oenocytes and fat body and adult lantern (27–31).

This present study establishes that peroxisomes of size, shape, and centrifugal behavior like the peroxisomes of vertebrates occur abundantly in the adult Malpighian tubule and gut of *Drosophila melanogaster*. Furthermore, the activity of the peroxisomal "marker" enzyme, catalase, is cytochemically localized only to such organelles. Catalase activity previously had been measured in homogenates of abdomens of *Drosophila* but was not associated with a subcellular fraction (32). The present study demonstrates that the catalase is peroxisomal.

We also have demonstrated that the xanthine oxidase activity in Malpighian tubule in wild-type *Drosophila* is peroxisomal. One previous light microscopic study found xanthine dehydrogenase in the abdomen of wild-type *Drosophila* but neither localized this enzyme activity to specific tissues nor demonstrated the oxidase activity [I. Dickinson (University of Utah), personal communication].

The absence of cytochemically demonstrable xanthine oxidase in the rosy-506 mutant establishes peroxisomes of Malpighian tubule as a site at which the structural mutation of the rosy-506 mutant is expressed. The apparent effects on catalase activity that we found were less expected, although they might have been anticipated from the experience with certain human disorders in which single gene mutations seem to have multiple effects on peroxisomes (2–4). Several possible explanations for the effects of the rosy mutation on catalase need exploring. The rosy-506 mutation might affect peroxisomes in such a way as to alter their responses to the cytochemical and biochemical manipulations of this study. For instance, in the mutant, peroxisomes might be more readily disrupted by homogenization. The mutation might alter the peroxisomal membrane either to make it more leaky or to prevent the import of enzymes (33). More speculatively, there could be metabolic links through which the xanthine oxidase enzyme might affect catalase. Some authors posit involvement of xanthine dehydrogenase in iron metabolism, which could influence the availability of heme groups for catalase assembly (8, 34). Or, despite the fact that the rosy-506 mutant is derived from the same background stock as the wild type, it may be that genetic effects in the rosy strain influence catalase directly via presently unknown paths.

Whatever the ultimate explanation of our findings, this study identifies a mutation affecting peroxisomes in an animal convenient for genetic study.

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