The cytochemical demonstration of catalase and D-amino acid oxidase in the microbodies of teleost kidney cells

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Synopsis. The distribution of catalase and D-amino acid oxidase, marker enzymes for peroxisomes, was determined cytochemically in the kidney tubules of an euryhaline teleost, the three-spined stickleback.

Catalase activity was localized with the diaminobenzidine technique. The presence of D-amino acid oxidase was determined using $H_2O_2$ generated by the enzyme, D-alanine as a substrate, and cerous ions for the formation of an electron-dense precipitate. Both enzymes appeared to be located in microbodies. The combined presence of these enzymes characterizes the microbodies as peroxisomes. Biochemically and cytochemically, no urate oxidase or glycolate-oxidizing L-$\alpha$-hydroxy acid oxidase could be demonstrated.

Stereological analysis of the epithelia lining the renal tubules showed that the fractional volume of the microbodies is 5 to 10 times higher in the cells of the second proximal tubules than in the other nephronic segments or the ureter. The fractional volume of the microbodies was similar in kidneys of freshwater and seawater fishes.

Introduction

Vertebrate kidney cells are characterized by high numbers of peroxisomes (cf. Hruban & Rechcigl, 1969). These organelles are defined biochemically as small bodies containing one or more oxidases generating $H_2O_2$ and catalase, which is able to destroy $H_2O_2$ (de Duve, 1965, 1969; de Duve & Baudhuin, 1966). In the electron microscope, peroxisomes are revealed as small membrane-bound spherical structures, microbodies, with a diameter usually varying between 0.2 and 0.8 $\mu$m. The matrix is rather electron-dense and may contain crystalline inclusions (Hruban & Rechcigl, 1969).

Small organelles with the structural appearance of microbodies have been described in the kidney cells of several teleost species (Bulger, 1965; Trump & Bulger, 1967, 1968; Gritzka, 1963), including the stickleback *Gasterosteus aculeatus* (Wendelaar Bonga, 1973). To our knowledge, these bodies have been identified as peroxisomes by either cytochemical or biochemical techniques. Cytochemical evidence for the association of

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catalase with one or more H₂O₂-producing oxidases has so far mainly been presented for some mammalian organs, especially liver and kidney (Hruban & Rechcigl, 1969; Hruban et al., 1972; Hand, 1975). This is largely due to the relative scarcity of peroxisomes in organs other than liver and kidney, and the absence of a reliable technique for the histochemical localization of H₂O₂-producing oxidases. The application of the method for L-α-hydroxy acid oxidase developed by Shnitka & Talibi (1971) has not always been successful in the hands of other investigators (Novikoff & Novikoff, 1972; Reddy & Svoboda, 1972). The modified diaminobenzidine method as used by Briggs et al., (1975a) and van Dijken et al., (1975) reveals the presence of oxidase activities but does not allow the localization of the enzymes to be determined with any precision.

This paper deals with the microbodies in the renal tubules of sticklebacks of the euryhaline form *trachurus*. These fishes migrate in spring from the sea to fresh water and return in autumn to the sea. The distribution of the microbodies was studied by stereological methods in such a way that the fractional volume of these organelles in the cells of the various segments of the nephronic tubules and the ureters could be determined. The volume of the microbodies was compared in freshwater and seawater fishes.

The microbodies were identified as peroxisomes by the cytochemical demonstration, at the ultrastructural level, of catalase and D-amino acid oxidase activity. The localization of catalase was established by the diaminobenzidine (DAB) procedure (Novikoff & Goldfischer, 1969). For the localization of D-amino acid oxidase, the cerium technique recently developed by Briggs et al. (1975b) for the demonstration of NADH-oxidase on the surface of the outer cell membrane of human polymorphonuclear leucocytes was used. This procedure is based on the formation of an electron-dense precipitate during incubation with cerous ions and in the presence of aminotriazole to inhibit endogenous catalase. The cerous ions form an insoluble compound, presumably cerium perhydroxide, with the H₂O₂ generated by the oxidase. A valid localization of intracellular oxidase activity may be expected if the substrate used is able to penetrate the cytoplasm and cytoplasmic organelles.

**Material and methods**

Adult female stickbacks of the migrating form (*Gasterosteus aculeatus trachurus*) were obtained from freshwater canals and from the sea. Body length varied between 60 and 65 mm. The fishes were kept in the laboratory for at least one month in aquaria with running tap water or with natural seawater at 15°C and a daily light period of 8 h.

For the morphometric studies, the kidneys were fixed for 2 h in a freshly-prepared mixture of glutaraldehyde (3%) and osmium tetroxide (1%) in phosphate buffer (0.1 M; pH 7.4) at 0°C. The fractional volume of the microbodies was determined by lineal integration (Loud et al., 1965). A square grid of sampling lines at a distance of 5 mm was projected on electron micrographs at a final magnification of × 20 000. Only circular cross-sections of the renal tubules were used for morphometric analyses. The nuclei were excluded from the sampling area. A length of 2000 µm per cell type was sampled in each of the five animals examined per group. Student's *t*-test was used for statistical analysis.

For the demonstration of catalase, small kidney blocks were prefixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 1 mM CaCl₂ and 1 mM MgCl₂, for 30 min at 0°C. The blocks were washed 5 times in 0.1 M tris-HCl buffer
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(pH 8.5) and incubated in 5 ml freshly-prepared medium containing 0.06% \(\text{H}_2\text{O}_2\) and 10 mg 3,3-diaminobenzidine in 0.1 M tris-HCl buffer (pH 8.5) for 90 min at 37°C. Unfixed tissue was washed only once before incubation. In control experiments, glutaraldehyde-fixed tissue was pre-incubated for 30 min in 0.1 M tris-HCl buffer (pH 8.5) in the presence of 0.05 M 3-amino-1,2,4-triazole and subsequently incubated with the same concentration of this inhibitor.

In order to select a suitable substrate for the cytochemical demonstration of oxidase activity, the capacity of the tissue to oxidize L-lactate, glycolate, D-alanine, sodium urate, and uric acid was assayed biochemically. Oxygen uptake rates of crude kidney homogenates, suspended in 0.1 M phosphate buffer (pH 7.3), were determined with a Clarke oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co.).

For the cytochemical localization of D-amino acid oxidase, the method developed by Briggs et al. (1975b) was modified. Unfixed tissue, as well as tissue prefixed for 10 min at 0°C in either 3% glutaraldehyde or 3% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M cacodylate buffer (pH 7.2), was pre-incubated in 0.1 M tris-maleate buffer (pH 7.5) containing 50 mM aminotriazole and 5 mM CeCl\(_3\) for 30 min at 25°C, and incubated in the same medium containing 50 mM L-lactate, glycolate, or D-alanine, or saturated with uric acid or sodium urate, for 2 h at 25°C under continuous aeration. After incubation the tissue blocks were transferred to 0.1 M cacodylate buffer (pH 6.0) to remove any cerium hydroxyde formed during incubation. In control experiments, oxygen-free nitrogen was led through the media during the incubations.

After the cytochemical staining procedures, the tissue blocks were washed once in cacodylate buffer (pH 7.2) and post-fixed in a mixture of osmium tetroxide (1%) and potassium dichromate (2.5%) in the same buffer for 30 min at 0°C. After dehydration in an alcohol series, the blocks were embedded in Epon 812. Ultrathin sections, cut on a LKB-Ulrotome with a diamond knife, were examined unstained in a Philips EM 300 electron microscope.

Results

The nephronic tubules of sticklebacks consist of two major segments with a brush border, the first and second proximal segments, and a third segment without a brush border, the collecting tubule. The structure of the ureters is similar to that of the collecting tubules. All epithelial cells lining the nephronic tubules and the ureters are characterized by the presence of a well-developed basal labyrinth. The ultrastructure of these cells has been described previously (Wendelaar Bonga, 1973; Wendelaar Bonga & Veenhuis, 1974). Microbodies are present in all epithelial cell types. They are concentrated in the upper half of the cells. The diameters vary between 0.2 and 0.5 \(\mu\)m. They are generally smaller than lysosomes and differ from the latter organelles by the thickness of the limiting membrane (6–7 nm for peroxisomes, compared with 9–10 nm for lysosomes). In contrast to the matrix of lysosomes, which may contain membranous or particulate inclusions, the matrix of peroxisomes is homogeneously electron-dense. Crystalline inclusions were not observed. Continuity between the limiting membrane of the microbodies and the nuclear envelope or the endoplasmic reticulum, as reported for other tissues, (Novikoff & Novikoff, 1972; Hruban et al., 1972; Pipan & Pšeničník, 1975), was not seen either.

The distribution of the microbodies in the kidney was estimated by linear analysis of electron micrographs (Loud et al., 1965) of kidney tubules of freshwater and
seawater fishes. The volume of the bodies, as a percentage of the cytoplasmic volume of the cells, is equally low in the first proximal tubules, the collecting tubules, and the ureters. In the cells of the second proximal tubules, on the other hand, the percentual volume of microbodies is five to ten times higher. There are no statistically significant differences between freshwater and seawater adapted fishes (Fig. 1). The number of microbodies in the cells of the very short nephronic neck segment (this segment was not analysed stereologically because of its small size) was not unlike that in the cells of the first proximal tubule.

In unfixed tissue exposed to DAB and H$_2$O$_2$ for the localization of catalase, the microbodies were hardly stained (Fig. 4). In these preparations some staining deposits were located on the mitochondria. The preservation of the cell structure was very poor. After fixation with glutaraldehyde prior to incubation with DAB and H$_2$O$_2$, the microbodies were heavily stained with an electron dense precipitate (Figs. 2 & 3). This fine granular precipitate was evenly distributed in the matrix of the microbodies. It was confined to these bodies only. It was not found in association with the nuclear envelope, endoplasmic reticulum or mitochondrial cell components known to exhibit DAB oxidation in other tissues (Widmann et al., 1972; Roels et al., 1975). When H$_2$O$_2$ was omitted, no precipitate was formed (Fig. 5). Precipitate was also absent from microbodies in control tissue (Fig. 6) incubated in the presence of H$_2$O$_2$ and aminotriazole, an inhibitor of the peroxidatic activity of catalase (Novikoff & Goldfischer, 1969).

Measurements of oxygen consumption during incubation of kidney homogenates showed that the renal tissue was able to oxidize D-alanine. No oxidation of glycolate or urate could be demonstrated. In tissue sections prepared after incubation, in the presence of cerous ions with urate and glycolate as substrates and aminotriazole to inhibit catalase activity, no staining of microbodies was observed. However, with D-alanine as substrate, concentrated staining deposits were distributed evenly in the matrix of the microbodies (Fig. 7). Prefixation with glutaraldehyde or formaldehyde did not notably influence the intensity of staining. Cellular preservation, very poor without prefixation (Fig. 8), was greatly improved, although still not entirely satisfactorily, after short prefixation with glutaraldehyde (Fig. 7) or formaldehyde (Fig. 9).

In 1 µm-thick plastic sections examined in the light microscope, the microbodies appeared as small brownish particles. At the ultrastructural level, small deposits were
Figures 2, 3 & 4 show cells of the second proximal tubules, incubated with DAB and H$_2$O$_2$ for the demonstration of catalase activity. Abbreviations for Figures 2–9: d = desmosome; n = nucleus; m = mitochondrion; p = peroxisome. Bars = 1 μm.

**Figure 2.** Cells prefixed with glutaraldehyde, showing intensely stained peroxisomes.

**Figure 3.** Detail of cell prefixed with glutaraldehyde, showing intensely stained peroxisomes and unstained mitochondrion.

**Figure 4.** Detail of unfixed cell; the mitochondria show some reaction product, while the peroxisomes are hardly stained.
found outside the microbodies, mainly on parts of the mitochondrial envelope (Fig. 7) and occasionally on the outer cell membranes.

In controls, incubated in anaerobic conditions which prevent substrate oxidation, no precipitate was found. In aerobic conditions without oxidase substrate, the peroxisomes were unstained, while small cerium deposits were occasionally found on the outer cell membranes and the mitochondrial envelope.

All microbodies observed in the renal tubular cells reacted positively to DAB exposure as well as to the D-amino acid oxidase test. It is, therefore, concluded that the enzymatic activities demonstrated with these methods are located within the same type of organelle.

Discussion

The microbodies in the renal tubular cells of the stickleback kidney are able to oxidize DAB in the presence of H₂O₂. This is an indication for the localization of catalase within these bodies. Not only catalase but also peroxidases give positive results with the DAB procedure. However, the difference we found between the staining results in unfixed and prefixed tissue points to catalase. Without prefixation with glutaraldehyde, these bodies were heavily stained with reaction product. Glutaraldehyde is known to inhibit the catalatic activity of catalase. In contrast, the peroxidatic activity of catalase, and thus its capacity to oxidize DAB in the presence of H₂O₂, is greatly...
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Figures 7, 8 & 9 show cells of the second proximal tubules incubated with CeCl₃ and D-alanine, in the presence of aminotriazole, for the demonstration of D-amino acid oxidase activity.

**Figure 7.** Cells prefixed with glutaraldehyde showing intensely stained peroxisomes. Traces of reaction product are present on the mitochondrial membranes and between the lateral cell membranes; mv = multivesicular body.

**Figure 8.** Detail of an unfixed cell, showing positively stained peroxisomes; the mitochondrial envelopes, but not the cristae, are also stained.

**Figure 9.** Detail of a cell prefixed with formaldehyde; the peroxisomes are well stained.
enhanced by glutaraldehyde (Roels & Wisse, 1973; Herzog & Fahimi, 1974). The activity of many peroxidases is reduced by glutaraldehyde fixation, and also by the high concentration of hydrogen peroxide (0.06%) used in our study (Roels et al., 1975). It is, therefore, likely that it is catalase and not peroxidase that accounts for the positive reaction of the microbodies in the DAB procedure.

In unfixed kidney tubules some precipitate was found on the outer membranes and the cristae of the mitochondria. The reactivity of the mitochondria was completely suppressed by prefixation with glutaraldehyde. The capacity of mitochondria to oxidize DAB in unfixed tissue is well known. It is probably due to oxidation by the cytochrome c-cytochrome oxidase complex (Seligman et al., 1968; Roels, 1974).

The D-amino acid oxidase procedure resulted in heavy deposits of the cerium compound. The precipitate was almost exclusively located in the microbodies. This result shows that the cerium technique, developed by Briggs et al. (1975b) for the demonstration of NADH-oxidase on the surface of human leucocytes, is also applicable to the localization of intracellular oxidase activity. The substrate used in our experiments, D-alanine, is apparently capable of penetrating into microbodies, in unfixed as well as prefixed tissues. In our opinion this procedure is superior to other methods available for the cytochemical localization of \( \text{H}_2\text{O}_2 \)-generating oxidases at the ultrastructural level. The tetrazolium technique, for example, which has proved to be useful for the light microscopical demonstration of oxidase activity, is of no value in electron microscopy (Beard & Novikoff, 1969). Another technique, based on the enzymatic reduction of ferricyanide (Shnitka & Talibi, 1971), was originally used for the demonstration of L-\( \alpha \)-hydroxy acid oxidase in rat kidney peroxisomes, but some workers, using rat kidney and other mammalian tissues, have obtained negative results with it, although enzymatic activity was found to be present in homogenates (Novikoff & Novikoff, 1972; Reddy & Svéboda, 1972). The modified procedure used by Hand (1975) resulted in inhomogeneous staining of the microbodies and heavy random and non-specific precipitates. The staining result proved to be markedly influenced by prefixation in glutaraldehyde. Random background staining was also reported by Burke & Trelease (1975), who used a similar technique in a study of plant peroxisomes. With the cerium procedure we did not find random background staining in either unfixed tissue or tissue prefixed with glutaraldehyde or formaldehyde. Non-specific deposits were limited to some parts of the outer cell membranes and the mitochondrial envelopes.

An indirect method for the demonstration of \( \text{H}_2\text{O}_2 \)-generating oxidases was used by Briggs et al. (1975a) and van Dijken et al. (1975). These authors observed a positive reaction with the DAB procedure when \( \text{H}_2\text{O}_2 \) was replaced by an oxidase substrate. The staining reaction is probably due to oxidation of DAB by either peroxidase (Briggs et al., 1975a) or catalase (van Dijken et al., 1975), and \( \text{H}_2\text{O}_2 \) generated by the oxidase. As stated by the authors, this procedure results in the localization of peroxidase or catalase, and not necessarily of the oxidase. The possibility cannot be excluded that the \( \text{H}_2\text{O}_2 \) is generated outside the microbodies and enters these organelles by diffusion.

The D-amino acid oxidase activity found in the stickleback kidney was not notably inhibited by prefixation in glutaraldehyde or formaldehyde. Severe inhibition by glutaraldehyde, but not by formaldehyde, has been reported by Hand (1975) for rat liver L-\( \alpha \)-hydroxy acid oxidase.

D-amino acid oxidase is a characteristic enzyme of liver and kidney peroxisomes (Hruban & Rechcigl, 1969). As far as teleosts are concerned, it has been demonstrated biochemically in the kidneys of carp and trout (Sarlet et al., 1950). De Duve &
Baudhuin (1966) have suggested that the D-amino acids oxidized in the liver and kidney originate from intestinal bacteria.

We were unable to demonstrate urate and glycolate oxidation in stickleback kidney. Urate oxidase has so far only been found in peroxisomes of vertebrate liver and of protozoa (de Duve, 1969). The presence of enzymes able to oxidize shorter chain L-a-hydroxy acids like glycolate has been reported for the kidneys of various vertebrates although species specific differences are known. The enzyme has been demonstrated in pig kidney, but proved to be absent in the kidney of the rat (McGroarty et al., 1974).

The present demonstration of the association of catalase and D-amino acid oxidase in the microbodies of the stickleback kidney characterizes these organelles as peroxisomes (de Duve, 1965, 1969). Although microbodies have been described in the kidneys of several teleost species (Gritzka, 1963; Bulger, 1965; Trump & Bulger, 1968; Wendelaar Bonga, 1973), cytochemical evidence for their identity as peroxisomes has not been presented before.

Our stereological data show that these organelles are concentrated in the second proximal tubule. The fractional volume of these organelles in this segment is 5 to 10 times higher than that in the other nephronic segments and the ureters. Also in rodents (Beard & Novikoff, 1969; Jacobson & Jørgensen, 1973) and in the amphibian Bufo marinus (Roels et al., 1970), microbodies are more numerous in the terminal portion of the proximal tubules. The percentage volume of peroxisomes established in the stickleback second proximal tubule (0.5% i.e. 4—7 per 100 μm² of thin sections) is relatively low when compared to the proximal tubule in adult mice (about 35 per 100 μm²; Goeckerman & Vigil, 1975).

The true function of renal peroxisomes is not known, but it has been suggested that these organelles are involved in gluconeogenesis, purine catabolism, and lipid and steroid metabolism (de Duve & Baudhuin, 1966; de Duve, 1969; Reddy & Krishnakantha, 1975). The relatively high number of peroxisomes in the second proximal tubule indicate that these organelles are involved in one of the specific functions of this segment. This segment is regarded as the main site of nitrogen catabolism in the teleost kidney (Hickman & Trump, 1969). The function of the renal tubules in reabsorption and secretion of ions differs considerably between freshwater and seawater adapted fishes (Hickman & Trump, 1969). Because the percentage volume of the microbodies is similar in freshwater and seawater sticklebacks, there is no indication of a role of these organelles in renal ion transport processes.

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