MICROPEROXIDASE

An Ultrastructural Tracer of Low Molecular Weight

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INTRODUCTION

Ultrastructural tracers serve the important purpose of defining tissue or cellular compartments and their interconnecting channels. The ideal tracer substance should be (*a*) nontoxic and physiologically inert, (*b*) composed of uniform particles of known size, (*c*) immobilized in its in vivo position by fixation, (*d*) capable of being accurately localized when viewed with the electron microscope, and (*e*) demonstrable at low concentration.

In addition, small molecular size is essential for many applications. For example, channels as narrow as 20 A have been demonstrated with colloidal lanthanum (12), a tracer smaller than almost all others now in use. Because of its toxicity, however, colloidal lanthanum must generally be applied to fixed rather than living tissue specimens. This complicates the interpretation of observations made with this tracer, since the effects of fixation on pore size or channel width are almost unknown.

Several nontoxic tracers are available for electron microscopy, but they are larger than 20 A in diameter: for example, ferritin (diameter 100–120 A, mol wt 900,000), horseradish peroxidase (HRP, diameter 50–60 A, mol wt 40,000), and cytochrome *c* (diameter around 30 A, mol wt 12,000). (For discussions of these and other tracers, see references 10, 11, 16, 17, and 20.)

Clearly there is a need for an ultrastructural tracer that is small, nontoxic, capable of being accurately localized, and demonstrable at low concentration. I have proposed as a tracer a heme-peptide (Fig. 1) with a molecular weight of only 1900 (9). This tracer, which was given the name microperoxidase (MP), does not appear to be toxic at the concentrations used in tracer studies. Like HRP and cytochrome *c*, MP is demonstrated for the electron microscope through its peroxidatic activity, which on a weight basis is about 1/4 that of HRP and 150 times that of cytochrome *c*. MP is readily demonstrated at low concentration, and the localization is as good as that obtained with HRP.

This communication is a report on some physical and chemical properties of MP and on a striking difference between the distribution of MP and that of HRP when the two substances are administered to mice. In particular, MP penetrates into an extracellular compartment from which HRP is largely excluded. This finding is consistent with the smaller molecular size of MP and suggests that MP may be generally useful for demonstrating the existence of channels not revealed in the living state by other tracers.

MATERIALS AND METHODS

Preparation of MP

MP was prepared from cytochrome *c* by digestion with pepsin. The heme-peptide thus obtained was purified by ammonium sulfate fractionation and dialysis; the solution was adjusted to a pH of 7.3 and lyophilized. The procedure is described in detail elsewhere (9; cf. 24).

Solutions of MP were stored at −20°C, because they support the growth of bacteria and because under some conditions the solutions are reportedly unstable (13). Solutions were shielded from light whenever possible.

Animal Studies; Fixation

MP or HRP was injected intravenously or perfused through the cerebral ventricles of mice in procedures similar to those described previously (4, 5, 9). Unanesthetized mice weighing 20–25 g were injected intravenously over a period of 30–60 sec with 20 mg of MP or 10 mg of HRP (type VI; Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.5 ml of 0.9% NaCl solution. (In a few experiments the renal blood supply was tied off under anesthesia before injection of MP, in order to sustain higher concentrations in the blood.) No overt signs of toxicity were observed at these dosages of MP. The mice were killed 15–30 min later, and the brains were fixed by ventriculo-cisternal perfusion; 2 hr later the brains were removed and immersed in the same fixative (see below) for 18–24 hr. In other experiments a solution of MP or HRP (10 or 5 mg/ml, respectively, in Elliott's solution B; [3]) was administered by ventriculo-

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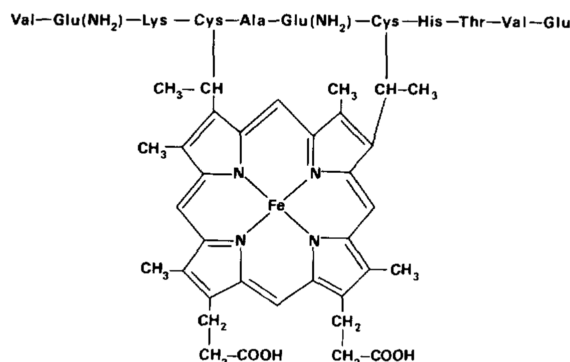

FIGURE 1 Structural formula of microperoxidase.

cisternal perfusion in anesthetized mice for 15–60 min; fixative was then perfused through the ventricles, and 2 hr later the brains were removed and immersed in fixative. Each observation reported here is based on the examination of specimens from three or more mice.

Specimens were fixed with a solution containing 5% glutaraldehyde, 4% formaldehyde (15), and 0.05% $CaCl_2$ in a 0.08 M cacodylate buffer of pH 7.2; the observations in the present report are based on this fixative. Good fixation and staining were also obtained with the following fixatives: (*a*) 6.5% glutaraldehyde in 0.08 M cacodylate buffer of pH 7.2, or (*b*) either of the above aldehyde fixatives to which trinitroresorcinol or trinitrocresol (14) had been added.

After fixation for 4 hr at room temperature or for 24 hr at 4°C, specimens were washed for 4–16 hr at 4°C in 0.2 M cacodylate buffer of pH 7.2. Sections 50 μ thick were cut on a Smith-Farquhar tissue chopper (Sorvall TC-2) and stored in 0.05 M tris buffer of pH 7.6 for 30–120 min before staining.

Peroxidase Staining

The 50 μ sections were stained by the method of Karnovsky (16) in a solution containing 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 0.05 M tris buffer of pH 7.6. Incubation of MP specimens was carried out with constant gentle swirling for 3 hr at room temperature. When incubation was prolonged much beyond 3 hr, there was an increase in background staining relative to "specific" staining.

After peroxidase staining, specimens were rinsed, postfixed in osmium tetroxide, dehydrated, and embedded in Araldite (Ciba [A. R. L.] Ltd., Duxford, Cambridge, England) by standard procedures. Some specimens were soaked in aqueous uranyl acetate solution after fixation with osmium tetroxide (4). Ultrathin sections were stained with lead citrate and examined with the AEI-6B electron microscope.

PROPERTIES OF MP; REVIEW OF LITERATURE

MP prepared as described is readily soluble in water and gives a deep red solution. A 4% solution at 23°C had a viscosity of 20 centipoises (about that of ethylene glycol).

Various properties of MP have been described by other investigators. (*a*) In an assay based on the oxidation of mesidine at pH 4.9, the peroxidatic activity of MP was found to be about 150 times that of cytochrome *c* and about 1/4 that of HRP (activities per unit weight) (21, 22; see also 1, 23). Relative peroxidatic activities are known to be highly dependent on substrate and on other conditions of the assay (18, p. 402; 19), so different values of the relative activities must be expected under the conditions used for staining tissue. (*b*) The isoelectric pH of MP has been estimated (7) to be 5.4. Thus MP bears a negative charge at physiological pH's. (*c*) The Soret absorption spectrum of MP has been reported (13).

OBSERVATIONS

Appearance of Reaction Product in Sections Containing MP

The appearance of the reaction product obtained with MP resembled closely that obtained with HRP, although the reaction product was sometimes more finely granular with MP. Apart from differences in intensity of staining, there were no consistent differences in the appearance of the reaction product when different methods of fixation and peroxidase staining were used.

In extracellular spaces that contained MP, there was often intensified staining in the basement lamina, around collagen fibrils, and on cell sur-

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faces. This observation suggests that MP is bound by some component of these structures.

In some places the intensity of staining was quite variable within a small region of extracellular space, for example, inside the circle in Fig. 2; in this region, a zone of intense staining adjoins a zone devoid of staining. There is no evidence to indicate whether the uneven distribution of reaction product reflects the true in vivo position of MP or is just an artifact. The same uneven distribution of reaction product occurs with HRP and can be seen in several previously published micrographs.

Distribution of MP and HRP in Brain

Stated briefly, the distribution of MP was the same as that of HRP when the two tracers were administered intravenously; when administered through the cerebral ventricles, the distribution was the same with one important exception. In the description that follows, the distribution of MP or HRP is assumed to be the same as that of the reaction product observed in thin sections. The full account of the distribution of MP will be presented in a forthcoming paper (Reese, Brightman, and Feder, in preparation).

After intravenous administration, both MP and HRP filled the capillary lumen and extended between capillary endothelial cells up to but not beyond the tight junction closest to the lumen (not illustrated; cf. 4, 9). The results were essentially the same with both tracers.

After intraventricular administration, HRP filled the interstitial space of the neuropil, extended

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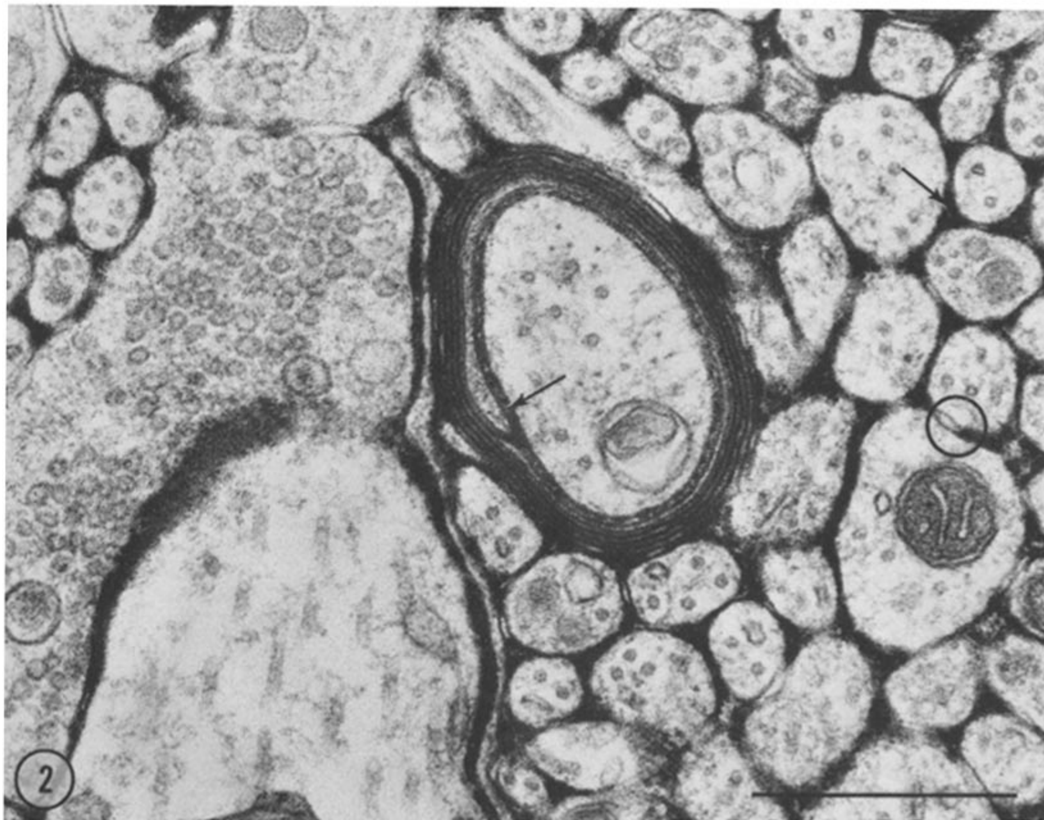

FIGURE 2 Cerebral cortex of mouse perfused in vivo through the cerebral ventricles with MP. As shown by the presence of reaction product, MP has passed from the interstitial space (arrow, upper right) into the periaxonal space (arrow, center). (The periaxonal space is largely devoid of tracer in parallel experiments with HRP; see text.) The uneven staining inside the circle is discussed in Observations. In this specimen, perfusion with MP solution was carried out for 15 min before the brain was fixed by perfusion. Scale bar is 0.5 μ. $\times$ 70,000. (Micrograph by Drs. T. S. Reese and M. W. Brightman.)

from this space across the region of the capillary basement lamina, and passed between capillary endothelial cells up to but not beyond the tight junction closest to the basement lamina (4). MP occupied the same spaces, but in addition it regularly passed into all parts of the periaxonal space—the extracellular compartment between the axon and sheath of myelinated fibers (Fig. 2). In particular, MP entered the long, "internal" part of the periaxonal space, that is, the part surrounded by compact myelin. HRP, in contrast, was rarely found inside the latter space, though it regularly penetrated a short distance from the node into the paranodal segment of the periaxonal space. The difference between MP and HRP in ability to penetrate into the periaxonal space was consistent and striking. Thus, in regions of average over-all staining intensity, MP was found in the periaxonal space of more than 80% of cross sections of myelinated fibers, whereas the corresponding figure for HRP (which was found almost entirely in the paranodal segment) was less than 5%.

DISCUSSION

A channel connecting the interstitial space and the periaxonal space in the mouse brain is revealed consistently and clearly by MP but only sporadically by HRP; this fact is presumably due to the difference in molecular size of the two tracers.

The dimensions of MP are not known, but they can be estimated easily. A nickel-etioporphyrin has been determined by X-ray crystallography to be about 10 A in diameter and 5 A thick (8, p. 6), and presumably the heme part of MP, which comprises about one third of the total weight of MP, is about the same size.

If the monomeric form of MP is assumed to be spherical, its molecular diameter can be estimated in two ways. From molecular weights and the reported radii of raffinose and ribonuclease (based on diffusion coefficients) (2), and from the assumption that the diameter varies as the cube root of the molecular weight, the diameter of MP (mol wt 1900) can be estimated by interpolation to be 20 A. Alternatively, a diameter of 17 A follows from the assumption that each MP molecule is a sphere with density the same as that of MP itself, i.e., that

$$V = (1/6)\pi d^3 = M/\rho N,$$

so that

$$d = (6M/\pi\rho N)^{1/3},$$

where V is the volume of an MP molecule, d is its diameter, M is the molecular weight of MP, ρ is the density of MP (assumed to be 1.3 g cm^{-3}, which is a density close to that of most proteins; [6]), and N is Avogadro's number. Because of the nature of the underlying assumptions, these calculated diameters obviously provide only a rough indication of the true dimensions of the MP molecule. Interpreting the behavior of MP as a tracer is further complicated by the fact that MP binds to proteins (Feder, unpublished observations; cf. 8, p. 25) and forms aggregates reversibly in solution (7, 13; Feder, unpublished observations). Thus, even knowledge of the actual dimensions of monomeric MP would be of very limited value for making predictions about the passage of MP through small pores in vivo.

Of the ultrastructural tracers that are nontoxic, accurately localized, and detectable at low concentration, MP has the lowest molecular weight. Although it is not possible to specify the actual size of MP under physiological conditions, MP has been shown in the present study to penetrate regularly into a compartment from which HRP is largely excluded. This finding suggests that MP may be generally useful for demonstrating the existence of channels not revealed in the living state by other tracers.

Microperoxidase and similar heme-peptides have other possible applications. If a method of conjugation can be devised, it should be possible to prepare a series of heme-labeled peptides and proteins that could serve as tracers of any desired size above a molecular weight of 2000. A method of conjugation would also make possible the preparation of MP-labeled antibodies, which could be used for immunocytological staining.

SUMMARY

A heme-peptide with a molecular weight of only 1900 has been found useful as an ultrastructural tracer. This compound, given the name microperoxidase, appears to be nontoxic, is demonstrable at low concentration through its peroxidatic activity, and is capable of being accurately localized in tissue sections. Microperoxidase administered in vivo penetrates into an extracellular compartment (the periaxonal space of myelinated fibers in mouse brain) from which horseradish

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peroxidase (mol wt 40,000) is largely excluded. This finding is consistent with the small molecular size of microperoxidase and suggests that this tracer may be generally useful for demonstrating the existence of channels not revealed in the living state by other tracers.

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