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CROSS-LINKING EXPERIMENTS FOR THE ELUCIDATION OF THE QUARTERNARY STRUCTURE OF CARBOXYLESTERASE IN THE MICROSOMAL MEMBRANE

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Rat liver microsomes have been labeled with a radioactive inhibitor, bis(4-nitrophenyl)phosphate, that binds specifically to carboxylesterases. After treatment of the labeled microsomes with dimethyl suberimidate and subsequent dodecyl sulfate electrophoresis the autoradiograms showed the same pattern of esterase oligomers as a control obtained with a purified trimeric carboxylesterase isoenzyme. Thus, the oligomeric structure of this enzyme is an inherent property and not an artefact occurring after solubilization from the membrane. No evidence was found for a close contact between carboxylesterase and other membrane proteins.

Little is known about protein-protein interactions in membranes of the endoplasmic reticulum. Chemical cross-linking reagents that are useful tools for the study of interactions between purified proteins cannot readily be used with membranes containing a large variety of differing proteins (1,2), due to the extreme difficulty in identification of the cross-linked components. However, if a specific label can be introduced into a certain membrane protein, it should be possible to identify the nearest neighbours of this protein.
Liver carboxylesterases (EC 3.1.1.1) occur mainly in the membranes of the endoplasmic reticulum (3) and can be specifically labeled by certain organophosphorus diesters (4). The major carboxylesterase of rat liver, an isoenzyme with pI 6.0 (5), has been described as a trimer (6). In this respect

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it is similar to many other carboxylesterases (3). However, the tendency to oligomerization is not a common property of all related esterases: e.g. purified ox liver esterase associates only at high concentrations (7). Therefore, we could not exclude the possibility that the many trimeric esterases might be artefacts in that the delipidated subunits aggregate spontaneously during solubilization from the membrane. This study was designed to elucidate the aggregation state of the membrane bound rat liver carboxylesterase with pI 6.0.

Materials and Methods

Preparation of labeled microsomes: 1 g of fresh liver from adult male Wistar rats was homogenized with 10 ml 0.25 M sucrose using a glass homogenizer with teflon pestle (Wheaton, Millville, N.J.). The suspension was adjusted to pH 8 and incubated for 10 min at 4°C with 100 µl of a 10 mM solution of bis(4-nitro[^{14}C]phenyl)phosphate (75 mCi/mg; Hoechst, Frankfurt, FRG) in water. Microsomes were then prepared by differential centrifugation (8). The 105000 x g(max)-sediment was resuspended (in 0.25 M sucrose containing 20 mM triethanolamine/HCl pH 8.5) so that 1 ml of the suspension corresponded to 1 g of fresh liver.

Labeled esterase: 1 mg of highly purified rat liver esterase pI 6.0 (5) in 250 µl Tris buffer pH 8.0 was treated for 10 min at 4°C with bis(4-nitro[^{14}C]phenyl)phosphate (20 µl of a 10 mM solution in water) resulting in an almost complete loss of esterase activity. Excess inhibitor was removed by microscale gel filtration (Sephadex G 50 superfine, equilibrated and eluted with 20 mM triethanolamine/HCl pH 8.5).
Cross-linking and electrophoresis: Dimethyl suberimidate was synthesized from suberonitrile according to the procedure of Davies and Stark (9). Varying volumes of labeled microsomes (14 mg protein/ml) or purified esterase (1.5 mg protein/ml), respectively, were incubated for 30 min at 20°C with freshly prepared solutions of dimethyl suberimidate (5 mg/ml) in 0.2 M triethanolamine/HCl buffer pH 8.5 containing 0.25 M sucrose. Optimal results were obtained with equal volumes of cross-

linking reagent and protein solution. Samples incubated under the same conditions, but without the suberimidate, served as controls. The reaction was terminated by heating the samples for 2 min at 95°C with a fivefold volume of a dodecyl sulfate solution containing (i) 2.5 % sodium dodecyl sulfate and 5 % mercaptoethanol in 60 mM Tris/HCl buffer pH 6.8, or (ii) 0.1 % sodium dodecyl sulfate and 0.1 % mercaptoethanol in 10 mM sodium phosphate buffer pH 7.2.

Polyacrylamide gel electrophoresis in 7.5 % slab gels was performed with (i) tris/glycine buffers according to Maurer (1o), (7.5 % separation gel in 0.375 M Tris/HCl buffer pH 8.8 containing 0.1 % dodecylsulfate; 4.5 % spacer gel in 0.125 M Tris/HCl buffer pH 6.8 containing 0.1 % dodecylsulfate; electrode buffer: 0.025 M Tris/0.20 M glycine pH 8.3 containing 1 % dodecyl sulfate); or (ii) phosphate buffers according to Weber et al. (11), (7.5 % separation gel in 0.1 M sodium phosphate buffer pH 7.2 containing 1 % dodecyl sulfate; same electrode buffer as in the gel). The gels were stained with 0.1 % Coomassie brilliant blue R 250 in 50 % (w/v) trichloroacetic acid/water and destained with 7.5 % acetic acid. For autoradiography the gels were dried on filter paper in vacuo and pressed on X-ray films (Osray T4, Agfa-Gevaert) for 3 weeks. Bovine serum albumin and RNA-polymerase from E.coli (both from Boehringer, Mannheim, FRG) served as molecular weight markers.

Results and Discussion

Rat liver microsomes contain various carboxylesterases and other hydrolases that are susceptible to covalent, irreversible inhibition by bis(4-nitrophenyl)phosphate (8,12). The bulk of these enzymes appears in the molecular weight range of about 60000 on dodecyl sulfate electrophoresis, (Fig. 1, lane A). On less exposed autoradiographs the minor bands of this figure disappear and the major band is resolved into two sharp lines that correspond to several esterases (5,8), mainly the hydrolases pI 5.6 and 6.0 according to our preliminary nomenclature (3,5). After purification, hydrolase pI 6.0 is the only

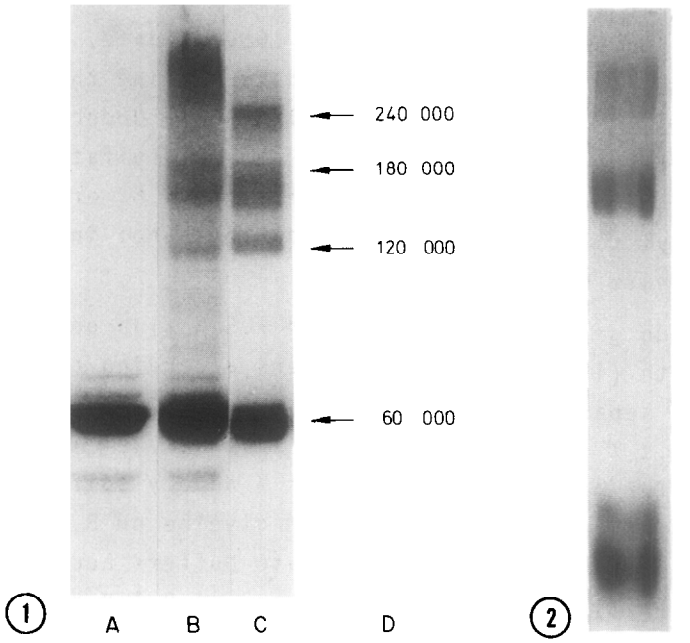

Fig. 1 Dodecyl sulfate electrophoresis in tris/glycine-buffer (10) of esterases and microsomes labeled with bis(4-nitro[^{14}C]phenyl)phosphate. (A)-(C): autoradiographs of the dried slab gels. (A), labeled microsomes, control. (B), labeled microsomes, cross-linked with dimethyl suberimidate. (C), labeled purified carboxylesterase pI 6.0, cross-linked under the same conditions as in (B). (D), M_r-values calculated from the relative mobilities of the marker proteins.

Fig. 2 Dodecyl sulfate electrophoresis in phosphate buffer (11) of purified carboxylesterase pI 6.0 cross-linked with dimethyl suberimidate. Slab gel, Coomassie stain.

microsomal carboxylesterase that occurs as a stable oligomer (3,6), all others are monomers or undergo reversible association (3,13).

Both freshly prepared rat liver microsomes and the purified hydrolase pI 6.0 were labeled with radioactive bis(4-nitrophenyl)phosphate. After subsequent cross-linking with the bifunctional reagent dimethyl suberimidate the proteins were

dissolved in concentrated dodecyl sulfate buffer and subjected to dodecyl sulfate electrophoresis in slab gels. The autoradiographs of the dried gels (Fig. 1, lanes B and C) show very similar patterns in the molecular weight range of 100000-200000. We conclude that the hydrolase pI 6.0 occurs in an identical state of association both in the microsomal membrane and after solubilization.

Obviously, the hydrolase pI 6.0 is not part of a well defined complex with other proteins of the microsomal membrane, e.g. of a multienzyme-complex. In such a case additional radioactive bands should be visible in Fig. 1B.

We were puzzled by the relatively strong band at a molecular weight of about 240000 visible after cross-linking of the purified hydrolase pI 6.0. This additional band might point to a tetrameric rather than the reported (3,6) trimeric structure of this enzyme. However, if the same mixture of crosslinked subunits was applied to flat or cylindrical gels and subjected to electrophoresis in a phosphate buffer, only three broad bands could be seen (Fig. 2). Our earlier result of three strong bands in cylindrical gels (6) could be reproduced with a polyacrylamide gel concentration of 5 % and the phosphate buffer.

We have no explanation for this discordance. However, this result shows that the cross-linking method for the study of oligomeric enzymes can only be handled very critically. Another observation should also be mentioned in this context: In purified pig liver carboxylesterase stable tetramers could be observed in addition to trimeric molecules (14). The dominant isoenzyme of pig liver carboxylesterase (15) resembles the hydrolase pI 6.0 both in specificity and in chemical properties.

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References

1. Freedman, R.B. (1979), Tr. Biochem. Sci. 4, 193-197.
2. Ji, T.H. (1979), Biochim. Biophys. Acta 559, 39-69.
3. Heymann, E. (1980) in "Enzymatic Basis of Detoxication", (Jakoby, W.B., ed.), Vol. 2, pp. 219-323, Academic Press, New York.
4. Krisch, K. (1971) in "The Enzymes", (Boyer, P.D., ed.), Vol. V, pp. 43-69, Academic Press, New York.
5. Mentlein, R., Heiland, S., and Heymann, E. (1980), Arch. Biochem. Biophys. 200, 547-559.
6. Arndt, R., Heymann, E., Junge, W., Krisch, K., and Hollandt, H. (1973). Eur. J. Biochem. 36, 120-128.
7. Junge, W., Krisch, K., and Hollandt, H. (1974), Eur. J. Biochem. 43, 379-389.
8. Heymann, E., Mentlein, R., Schmalz, R., Schwabe, C., and Wagenmann, F. (1979), Eur. J. Biochem. 102, 509-519.
9. Davies, G.E., and Stark, G.R. (1970), Proc. Natl. Acad. Sci. U.S.A. 66, 651-656.
10. Maurer, R. (1971), Disc. Electrophoresis, pp. 44-45, Springer-Verlag, Berlin, New York.
11. Weber, K., Pringle, J.R., and Osborn, M. (1972) in "Methods in Enzymology" (Hirs, C.H.W., and Timasheff, S.N., eds.), Vol. 26, pp. 3-27, Academic Press, New York, London.
12. Brandt, E., Heymann, E., and Mentlein, R. (1980), Biochem. Parmacol. 29, in press.
13. Arndt, R., Schlaak, H., Uschtrin, D., Südi, D., Michelssen, K., and Junge, W. (1978), Hoppe-Seyler's Z. Physiol. Chem. 359, 641-651.
14. Farb, D., and Jencks, W.P. (1980), Arch. Biochem. Biophys., in press.
15. Heymann, E., and Junge, W. (1979), Eur. J. Biochem. 95, 509-518.