ON THE TRANSIENT FORMATION OF AN ACETYL ENZYME INTERMEDIATE DURING THE HYDROLYSIS OF P-NITROPHENYL ACETATE BY PANCREATIC LIPASE

M. Sémériva, C. Chapus, C. Bovier-Lapierre and P. Desnuelle

Centre de Biochimie et de Biologie Moléculaire du C.N.R.S.,
31, Chemin Joseph-Aiguier, 13274 Marseille Cedex 2 (France)

Received April 16, 1974

SUMMARY : The hydrolysis of monomeric p-nitrophenyl acetate by lipase in the presence of 4 % acetonitrile involves, like that catalyzed by ordinary esterases and some proteases, an acylation and a deacylation step (here rate-limiting). A transient acetyl lipase intermediate can be isolated. Various interfaces were found to exert a considerable accelerating effect in the lipase-p-nitrophenyl acetate-acetonitrile system.

Introduction

Pancreatic lipase (glycerol ester hydrolase ; EC 3.1.1.3) has been known for years to hydrolyze at a very high rate emulsions of insoluble long chain fatty acid esters of glycerol and other alcohols (1). New perspectives in this field were opened by the more recent finding that lipase can also act on isotropic micellar dispersions of short chain glycerides (2) and, although with a reduced efficiency, on molecular solutions of monomeric glycerides in the presence of low concentrations of some organic, water-miscible compounds (3). If monomeric PNPA* could be shown to be also hydrolyzed in this latter system, current investigations on the mechanism of lipase-catalyzed reactions would be facilitated. PNPA is a substrate widely used for ordinary esterases and some proteases. Its main advantage over other substrates is to permit very sensitive activity determinations and also to give the possibility of carrying out these determinations simultaneously by spectrophotometry and titrimetry. Enzyme kinetic results obtained with monomeric substrates are also much simpler to interpret than those yielded by emulsified or micellar systems. All assays reported below were performed with the aid of PNPA solutions in 4 %

* Unusual abbreviation, PNPA, p-nitrophenyl acetate.

acetonitrile. The complete absence of aggregates in these solutions has been carefully checked by an already described technique (3).

Materials and Methods

Porcine pancreatic lipase preparations obtained by the method of Verger et al (4) were used without further purification. PNPA, an Aldrich product (Milwaukee, USA) was crystallized twice from absolute ethanol. All other reagents were purchased from Fluka (Zürich, Switzerland) and were of the best available grade. Tritium-labelled PNPA was prepared by esterification of p-nitrophenol by $[^3H]$ acetic acid (CEA, Saclay, France ; specific radioactivity, 25 mCi/mmole) in the presence of dicyclohexyl carbodiimide in dry ethyl acetate (5). A pure product was obtained after 2 crystallizations from absolute ethanol with an overall yield of 50 % (calculated from acetic acid). Its specific radioactivity was similar to that of the original acetic acid sample (25 mCi/mmole).

As already mentioned before, the lipase-catalyzed PNPA hydrolysis was followed kinetically at 25° C and pH 7.5, either by spectrophotometry (400 nm ; Zeiss recording Spectrophotometer) or by titrimetry (Radiometer recording pH-stat). The molar concentration of the lipase solutions was calculated from their absorbance at 280 nm, assuming for the enzyme an extinction coefficient ($E^{1\,\%}_{1\,cm}$) of 13.3 and a molecular weight of 50,000.

Results and Discussion

Time course of PNPA hydrolysis by lipase.

Fig. 1 shows that monomeric PNPA is hydrolyzed by lipase in the presence of 4 % of acetonitrile. In addition, the release of p-nitrophenol starts very fast, slows down exponentially and then becomes linear with time, indicating the existence of a steady state. Extrapolation to zero time of the linear part of the curve shows that the amount of p-nitrophenol liberated during the exponential phase (the "burst") is practically one mole per mole of lipase. The burst and the steady state rate are proportional to the enzyme concentration. It is noteworthy that the shape of the curve in fig. 1 and further kinetic

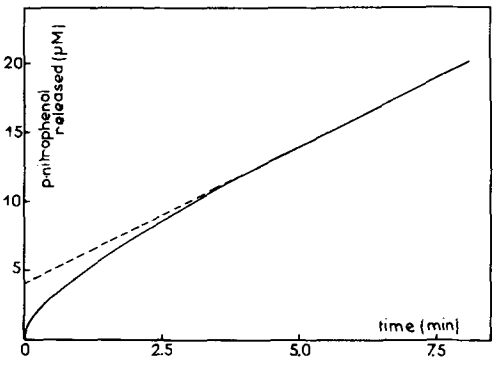

Fig. 1 : Kinetic aspects of p-nitrophenol release from PNPA by pancreatic lipase. A 3.12 mM PNPA solution in 4 % acetonitrile and a 80 mM Tris-HCl buffer (pH 7.5) was incubated with lipase (final concentration, 4.5 μM). The original tracing given by the recording spectrophotometer is reproduced in the fig.

studies to be published in a forthcoming paper are fully consistent with the model first described by Hartley and Kilby (6) and Bender and Kezdy (7) for the hydrolysis of PNPA by ∝ chymotrypsin. Since the rate-limiting step in the PNPA-lipase-acetonitrile system appears to be the deacylation of a transient acetyl-lipase derivative, this latter should accumulate during the pre-steady state period and its isolation should be possible if the reaction is quenched during the steady state period under conditions insuring to the derivative a good stability.

Characterization of the $[^3H]$ acetyl lipase intermediate.

In order to avoid side-reactions due to unspecific binding of PNPA to lipase, it was found necessary to saturate the corresponding sites by first incubating the enzyme (3 mg/ml solution in a 80 mM Tris-HCl buffer at pH 7.5) for 90 min. at 25° C with 2.5 mM unlabelled PNPA. The PNPA concentration was held constant during the assay by 2 additions of 2.5 μmoles of the reagent per ml of solution after 30 and 60 min. The resulting derivative isolated by Sephadex G 25 filtration in a 0.1 M Tris-HCl buffer at pH 7.5 was found to possess a fully functional active site towards emulsified tributyrin and monomeric PNPA in the presence of acetonitrile. Consequently, the derivative was suitable for the labelling of this site by a further incubation at pH 7.5 and 25° C

with [^{3}H] PNPA (concentration of the derivative, 2 mg/ml ; of PNPA, 2.5 mM). After exactly 3 min., the pH of the reaction mixture was lowered to 5.0 by addition of a 1 M sodium acetate buffer at pH 4.5. As shown by fig. 2a, a Sephadex filtration at pH 5.0 and 0° C of the resulting solution readily separated unreacted PNPA and p-nitrophenol from a lipase derivative containing one labelled acetyl radical per mole ([^{3}H] acetyl lipase). No traces of PNPA and p-nitrophenol could be detected under the protein peak, confirming that the radioactivity was entirely carried by the acetyl radical. Moreover, this radical was found to be stable at pH 5.0, as shown by the fact that no radioactivity loss occurred upon re-chromatography of the material under the same conditions. It was noteworthy that the stability of the derivative was not due to enzyme denaturation, since a complete deacylation (fig. 2b) was observed to result from an increase to 7.5 of the pH of the solution. Therefore, the acylation and deacylation steps could be considered fully reversible.

Accelerating effect of interfaces.

Since the lipase activity on monomeric PNPA in the presence of 4 % acetonitrile is low as compared to that on ordinary emulsified substrates, the specificity of this sort of activity may be questioned. A first approach to this important problem is to note that the 2 processes have in common the property to be considerably accelerated by interfaces. For example, 700 mg of glass beads (Solid Support Aerograph, 170-230 mesh ; mean diameter, 75 ± 15 μ) were siliconized according to Brockman et al (8) and added to 7 ml of a mixture 3.12 mM in PNPA, 4 % in acetonitrile, 1 mM in Tris-HCl pH 7.5 and 0.1 M in NaCl. The reaction rate observed in a pH-stat under stirring was 500-fold higher than without beads. A similar effect of glass beads on the hydrolysis of a triglyceride substrate (tripropionin) by lipase in an aqueous system has recently been reported (8). In the same connection, 0.7 ml of dodecane was emulsified in 4 ml of 3.2 mM taurocholate. Addition of 1 ml of this emulsion to 14 ml of a mixture containing 0.5 mM PNPA, 80 mM Tris-HCl at pH 7.5, 1.5 mM sodium taurocholate 0.1 M NaCl and 10 mM $CaCl_2$ was found to increase the rate

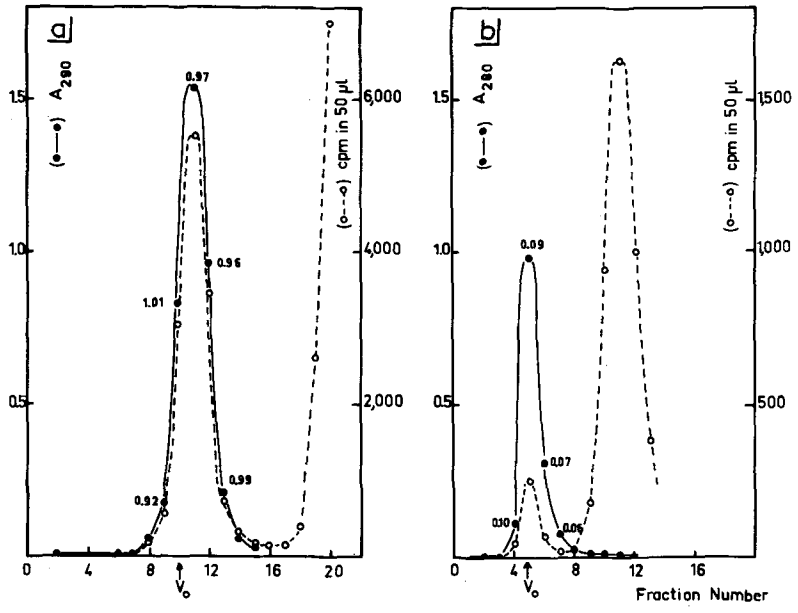

Fig. 2 : Acylation and deacylation of lipase. Fig. 2a : Acylation step. The labelled lipase derivative resulting from treatment with [^{3}H] PNPA (see text) was filtered at 0° C through a Sephadex G 25 column (0.9 x 21 cm) equilibrated and eluted with a 0.1 M sodium acetate buffer at pH 5.0. Radioactivity was counted in 50 µl aliquots with the aid of a Packard Liquid Scintillator. The numbers along the protein peak indicate the amount of [^{3}H] acetyl groups bound per mole of lipase. Fig. 2b : Deacylation of the above derivative (1 mg/ml) by a 30 min. incubation at pH 7.5 and 25° C in a 0.1 M Tris-HCl buffer. The conditions of the gel filtration and the symbols are the same as in fig. 2a, except for the dimensions of the column (0.9 x 13 cm).

of the PNPA hydrolysis by a factor of 100. Moreover, the complex between lipase and large micelles designated "fast" lipase by Sarda et al (9) because of its abnormal behavior on Sephadex could be shown to be 300 times as active as lipase alone in a system similar to those described above. The additional interest of this latter assay is to indicate that the action of lipase on dissolved substrates is enhanced, not only by the presence of an interface in the system, but also by a previous binding of the enzyme to an interface.

Finally, the influence exerted on the hydrolysis rate by the air-water and glass (or polyethylene)-water interfaces inherent to any system used for testing lipase activity (10, 3) could be confirmed here by taking advantage of

the 2 techniques (spectrophotometric and titrimetric) that can be employed to follow PNPA hydrolysis. These techniques should normally lead to the same results throughout the steady state period. In fact, results obtained by titrimetry were 150 fold higher. This large difference was attributed to the fact that interfaces are much smaller in a spectrophotometer cuvette than in a pH-stat beaker (including stirrer and sometimes air bubbles). The interface effects reported above can be expexted to accelerate the deacylation step which is rate-limiting in the lipase-catalyzed reaction.

Acknowledgements

Financial help from Délégation Générale à la Recherche Scientifique et Technique (Comité : Activation Sélective en Chimie Organique) and technical assistance from Mrs. F. Ferrato for the preparation of lipase are gratefully acknowledged.

References

1. Desnuelle, P. (1972) in the Enzymes, Vol. VII, p. 575, 3rd Edn, (Boyer, P.D., ed.), Academic Press, New-York.
2. Entressangles, B. and Desnuelle, P. (1968), Biochim. Biophys. Acta, 159,285.
3. Entressangles, B. and Desnuelle, P. (1974), Biochim. Biophys. Acta, in press.
4. Verger, R., de Haas, G.H., Sarda, L. and Desnuelle, P. (1969) Biochim. Biophys. Acta 188, 272.
5. Bodansky, M. and du Vigneaud, V. (1959) J. Am. Chem. Soc. 81, 5688.
6. Hartley, B.S. and Kilby, B.A. (1954) Biochem. J. 56, 288.
7. Bender, M.L. and Kézdy, F.J. (1964) J. Am. Chem. Soc. 86, 3704.
8. Brockman, H.L., Law, J.H. and Kézdy, F.J. (1973) J. Biol. Chem. 248, 4965.
9. Sarda, L., Maylié, M.F., Roger, J. and Desnuelle, P. (1964) Biochim. Biophys. Acta 92, 319.
10. Sémériva, M., Dufour, C. and Desnuelle, P. (1971) Biochemistry 10, 2143.