Irreversible inhibition of pancreatic lipase by bis-*p*-nitrophenyl methylphosphonate

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The reaction of porcine pancreatic lipase with an organophosphorus compound bis-*p*-nitrophenyl methylphosphonate (BNMP) resulted in the complete and irreversible inhibition of lipase activity on tributyrin emulsion (25°C, pH 7.5, 40 mM Na-veronal-HCl buffer) whereas the activity of the enzyme on *p*-nitrophenyl acetate solution remained unchanged. The BNMP-modified enzyme did not bind on hydrophobic interfaces (siliconized glass beads). Tyr 49 was presumed to be the modification site, and the conclusion has been made that this residue is implicated in the interface recognition site of pancreatic lipase.

Pancreatic lipase *Organophosphorus compound* *Enzyme modification* *Enzyme inhibition*

1. INTRODUCTION

Organophosphorus compounds, commonly used as the specific inhibitors of serine hydrolases, did not abolish pancreatic lipase activity in true solution [1]. The inhibition of the enzyme lipolytic activity has been achieved only by using the emulsions of these compounds, diethyl *p*-nitrophenylphosphate in particular [2], or the same compound in the presence of bile salt micelles and colipase [2,3]. A single serine residue, recently identified as Ser 152 [4], has been modified as the result of the reaction. The modified enzyme was inactive on emulsified triglyceride substrates [2] and did not bind on hydrophobic interfaces, i.e., siliconized glass beads, but retained the activity on *p*-nitrophenyl acetate solutions [5]. It was concluded that Ser 152 residue is implicated in binding the enzyme on hydrophobic interfaces [5].

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Abbreviations: DFP, diisopropyl fluorophosphate; DP, diisopropylphosphoryl group; BNMP, bis-p-nitrophenyl methylphosphonate

DFP, another common reagent of this type, was found to react selectively with lipase in true solution modifying Tyr 49 in the enzyme molecule [2,6]. As this modification was found to be nonessential for lipase activity towards tributyrin emulsion [2], no attempts were made to further study the role of this reactive group in lipase activity.

The present study shows that the reaction of porcine pancreatic lipase with BNMP solution resulted in complete and irreversible inhibition of the enzyme lipolytic activity. The inhibited enzyme did not bind on siliconized glass beads but retained the activity towards *p*-nitrophenyl acetate solution. As the modification of lipase with DFP completely prevented the reaction of the enzyme with BNMP, Tyr 49 side chain was presumed to be the reaction site for BNMP as well.

2. MATERIALS AND METHODS

Porcine pancreatic lipase was prepared as in [7], except that a Sephadex G-100 superfine column with an upward flow (Pharmacia K 100/100, Sweden) was used instead of the Sephadex G-100

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fine column used in the original method. The mixture of isoenzymes L_A and L_B obtained after further purification on a Whatman DE 52 column was found to be homogeneous on SDS disc electrophoresis [8] and essentially free from colipase as in [9].

BNMP was synthesized as in [10]. DFP from Fluka was redistilled under reduced pressure. [^{3}H]DFP with the radiochemical purity of 92% was obtained from Amersham. Commercial tributyrin (Reakhim, USSR) was fractionally distilled under reduced pressure and stored in sealed ampoules under argon at −20°C. *p*-Nitrophenyl acetate was prepared as in [11] and recrystallized twice from absolute ethanol. Siliconized glass beads were from Serva.

Lipase activity on unstabilized tributyrin emulsion was routinely measured on a pH-stat (TTT2/SBR3/ABU12, Radiometer, Denmark) as in [12]. The reaction of lipase with BNMP in large excess was followed either by the loss of the enzyme activity, or spectrophotometrically (Beckman UV 5260) by liberation of the product of the reaction, *p*-nitrophenol, at 25.0°C (pH 7.5) in 40 mM Na-veronal-HCl buffer solution. For reasons not understood, the progressive amounts of lipase, insensitive to BNMP, accumulated in the enzyme stock solutions during the prolonged standing. The ability of these 'aged' solutions to react with BNMP was restored by treating the enzyme with 0.5 M hydroxylamine solution at pH 7.5–8.0 for 2 h. Hydroxylamine was removed from the enzyme solutions on a Sephadex G-25 column.

In the residual activity measurements aliquots were withdrawn from the reaction mixture containing 0.1 μM lipase and 5–100 μM BNMP and assayed for the enzyme activity. A considerable amount of the inhibitor (~10%/h) was spontaneously hydrolyzed during the time required to achieve the complete suppression of lipase activity. To compensate this decrease in the BNMP concentration, the extent of its hydrolysis was determined by measuring the amount of released *p*-nitrophenol, and calculated amounts of BNMP stock solution in absolute ethanol were added every 60 min. The final concentration of ethanol in the reaction mixture did not exceed 3.5%.

The first-order rate constants of the reaction, k_I, were determined from the dependence of residual activity logarithms (log A%) on the reaction time t (fig.1, inset) by the least squares method.

In the spectrophotometric measurements, 8–17.5 μl of 8.0 mM stock solution of BNMP was added to 3.0 ml of 10 μM lipase solution and the rate of *p*-nitrophenolate release was followed at 400 nm. The obtained kinetic curves were treated by the non-linear least squares method [13] using the kinetic equation:

$$A_\infty - A = (A_\infty - A_0)e^{-k_I t} + k_0 t \quad (1)$$

where A_0 and A_∞ are the initial and final absorbances, respectively, A is the absorbance at time t, k_I is the observed first-order rate constant of the enzyme inhibition reaction, and k_0 is the observed zero-order rate constant of hydrolysis of the inhibitor.

The second-order rate constant of the inhibition reaction, $k_i = k_I/[I]_0$, was determined from the dependence of K_i on the inhibitor concentration (fig.1) by the least squares method.

The kinetics of hydrolysis of *p*-nitrophenyl acetate by lipase and the adsorption of the native and BNMP-treated lipase on siliconized glass beads were determined as in [14] and [15], respectively.

The modification of lipase with DFP was performed as in [2]. A Rackbeta scintillation counter (LKB, Sweden) was used for radioactivity measurements.

3. RESULTS AND DISCUSSION

The inhibition of lipase activity by BNMP followed the first-order kinetics up to 60–80% of the reaction (fig.1, inset). The pseudo first-order inhibition rate constants, k_I, obtained from the residual activity measurements agreed well with those obtained from the spectrophotometric determinations. The resulting k_I vs BNMP concentration plot was linear (fig.1), up to the solubility limit of the compound indicating that the inhibition of the enzyme proceeded in true solution. The value of the bimolecular rate constant $k_i = 1.25 \pm 0.05$ $M^{-1} \cdot s^{-1}$ was calculated from the data presented in fig.1.

The proposed kinetic model for the spectrophotometric data treatment (eqn1) adequately described the experimental results as indicated by

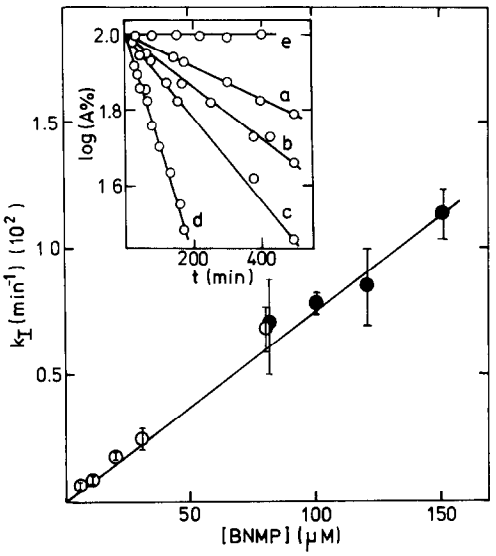


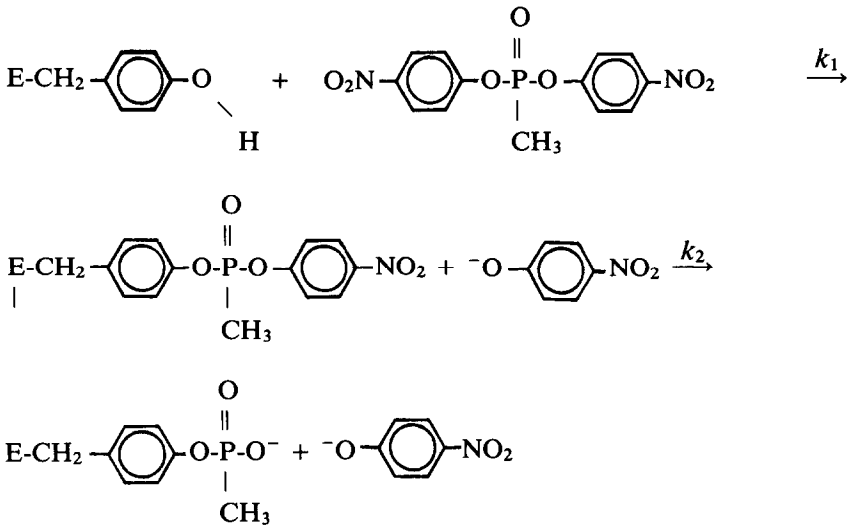
Fig.1. The kinetics of the inhibition of lipase activity on tributyrin emulsion by BNMP at 25°C in 40 mM Na-veronal-HCl buffer (pH 7.5). The dependence of the pseudo first-order rate constants on the BNMP concentrations. The data from spectrophotometric (●) and residual activity (○) measurements. Inset shows the time dependence of the inhibition of lipase activity by 10 μM (a), 20 μM (b), 30 μM (c) and 80 μM (d) solution of BNMP. Resistance of lipase initially modified by 50 mM DFP to the inhibition by BNMP (e) is also shown.

high correlation coefficients ($r \geqslant 0.999$), and the small and random deviation of the data from calculated curves. The amount of *p*-nitrophenol released in the pseudo first-order reaction of lipase with BNMP corresponded to the formation of 1.9 ± 0.2 mol/mol, and so the question arises whether the reaction of the inhibitor with the enzyme proceeded in two distinct reaction sites with equal rates or both *p*-nitrophenyl groups were released from the same inhibitor molecule. Two types of experiments were performed to clarify this question.

First, the reaction mixture was separated on the Sephadex G-25 column and further possible release of *p*-nitrophenol from the inhibited enzyme was examined. No additional *p*-nitrophenol release and/or recovery of the enzyme activity was observed during a long-term stay of the inhibited enzyme at pH 7.5, in 0.8 M hydroxylamine (pH 7.5) or 0.3 M KOH solutions.

Second, the ability of the DFP-modified enzyme [2] to react with BNMP was studied. The modification of lipase with the DFP solution resulted in a two-fold decrease in lipase activity on tributyrin emulsion and the incorporation of 1.09 mol/mol [^{3}H]DP into the enzyme molecule, which is in good agreement with earlier results [2]. The DFP-modified enzyme did not react with BNMP as indicated by the retention of the enzyme activity (fig.1, inset) and the lack of the first-order *p*-nitrophenol release. In accordance with these results the incorporation of [^{3}H]DP into the BNMP-modified lipase was not observed either.

From these experiments one may conclude that the reaction of BNMP with lipase proceeded at a single reaction site, at Tyr 49, as in the case of DFP, and that both *p*-nitrophenyl groups were released from the inhibitor molecule according to a formal reaction scheme:



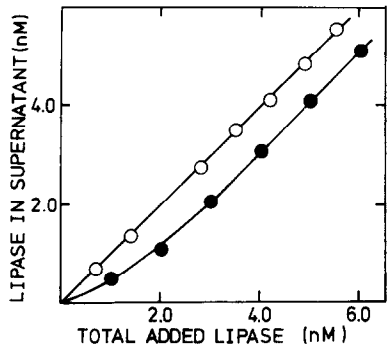


Fig.2. The lack of adsorption of BNMP-modified lipase on siliconized glass beads (○) at 25°C in 0.1 M Tris-HCl buffer (pH 7.5). Adsorption of the native lipase to the beads is shown (●) for comparison.

Since both *p*-nitrophenyl groups were released as a single kinetic event, the first stage in the scheme must be rate-limiting, i.e., $k_i = k_1$.

Although the mechanism of activation of the tyrosine hydroxyl group remains unknown at present, it is interesting to note that the rate constant of the reaction of lipase with BNMP is close to the value of $k = 1.48\ M^{-1} \cdot s^{-1}$ for the displacement of the *p*-nitrophenyl group from BNMP by *p*-methoxyphenolate anion [16], a reasonably good model for the tyrosine side chain.

It is also notable that the properties of the lipase derivative obtained after the reaction of the enzyme with BNMP are similar to those of the lipase derivative obtained after the reaction of diethyl-*p*-nitrophenylphosphate with its Ser 152 side chain (cf. [5]). In both cases, the modified enzyme retained the activity on *p*-nitrophenyl acetate solutions but did not bind on siliconized glass beads (fig.2, for lipase modified by BNMP). Probably the Tyr 49, as the Ser 152 residue (cf. [5]), is directly implicated in binding the enzyme on hydrophobic interfaces. As the modification of lipase with DFP did not result in great changes in the enzyme lipolytic activity, it can be suggested that the attachment of a charged, and hence highly hydrophilic methylphosphonate moiety to the Tyr 49 hydroxyl group greatly reduced the affinity of lipase for the substrate surface, and therefore depressed the enzyme lipolytic activity.

REFERENCES

[1] Sémériva, M. and Desnuelle, P. (1979) Adv. Enzymol. 48, 319–370.
[2] Maylié, M.F., Charles, M. and Desnuelle, P. (1972) Biochim. Biophys. Acta 276, 162–175.
[3] Rouard, M., Sari, H., Nurit, S., Entressangles, B. and Desnuelle, P. (1978) Biochim. Biophys. Acta 530, 227–235.
[4] Guidoni, A., Benkouka, F., De Caro, J. and Rovery, M. (1981) Biochim. Biophys. Acta 660, 148–150.
[5] Chapus, C. and Sémériva, M. (1976) Biochemistry 15, 4988–4991.
[6] Bianchetta, J.D., Bidaud, J., Guidoni, A.A., Bonicel, J.J. and Rovery, M. (1979) Eur. J. Biochem. 97, 395–405.
[7] Garner, C.W. jr and Smith, L.C. (1972) J. Biol. Chem. 247, 561–565.
[8] Laemmli, V.K. (1970) Nature 227, 680–685.
[9] Borgström, B. and Erlanson, C. (1973) Eur. J. Biochem. 37, 60–68.
[10] Behrman, E.J., Biallas, M.J., Brass, H.J., Edwards, J.O. and Isaks, M. (1970) J. Org. Chem. 35, 3063–3069.
[11] Kaufmann, A. (1909) Ber. 42, 3480–3483.
[12] Sémériva, M., Dufour, C. and Desnuelle, P. (1971) Biochemistry 10, 2143–2149.
[13] Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
[14] Sémériva, M., Chapus, C., Bovier-Lapierre, C. and Desnuelle, P. (1974) Biochem. Biophys. Res. Commun. 58, 808–813.
[15] Chapus, C., Sari, H., Sémériva, M. and Desnuelle, P. (1975) FEBS Lett. 58, 155–158.
[16] Brass, H.J. and Bender, M.L. (1972) J. Am. Chem. Soc. 94, 7421–7428.