Mechanism of Pancreatic Lipase Action. 2. Catalytic Properties of Modified Lipases†

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ABSTRACT: Reaction of lipase with diethyl pyrocarbonate results in the modification of three histidine residues. One is highly reactive, although without affecting the activity, while the two others react more slowly with a concomitant loss of activity on both dissolved and emulsified substrates. As previously shown, lipase can also be modified either by reaction of five carboxyl groups with carbodiimide (5N-lipase) or by esterification of one serine residue with diethyl *p*-nitrophenyl phosphate (DP-lipase). In the three cases, the activity on emulsified substrates is abolished. The modification of histidine residues results also in a loss of activity on dissolved substrates, suggesting that the essential histidine is at (or close to) the active site. The ability of lipase to be adsorbed on siliconized glass beads is not impaired in this reaction. By contrast, 5N-lipase is still able to hydrolyze dissolved monomeric substrates and to adsorb on siliconized glass beads. Therefore, the essential carboxyl group is assumed to play an important role in the interfacial activation. Finally, since DP-lipase is still fully active on dissolved *p*-nitrophenyl acetate, the serine residue, which has been previously suggested to be the acylable one, is more likely implicated in the recognition and the binding to interfaces, as confirmed by the inability of DP-lipase to be adsorbed on siliconized glass beads.

Chemical modifications of enzymes may lead to some important information concerning the mechanism of the catalyzed reaction and the structure–function relationship. Pancreatic lipase is not very easily specifically modified, probably because of its peculiar specificity (Sémériva and Desnuelle, 1976). For example, all attempts to obtain active site directed reagents for this enzyme have, so far, failed (Entressangles and Sémériva, unpublished results). Nevertheless, a few relatively specific chemical modifications have succeeded, including the reaction of a unique essential serine residue with E 600[1] (Maylié et al., 1972) and of five carboxyl groups with carbodiimides of which one is essential (Dufour et al., 1973). Modification of lipase by dye-sensitized photooxidation has suggested that a histidine residue is at (or near) the active center of the enzyme (Sémériva et al., 1971). However, several other residues, other than histidine, were modified by photooxidation, and, therefore, the first aim of this work was to seek for a more specific way of modifying histidine residues.

On the other hand, it seems difficult to attribute a precise role to these essential residues as long as only activity on emulsified substrates is measured. As discussed in the preceding paper of this issue, lipase adsorption to interfaces (Chapus et al., 1975), hydrolysis of dissolved monomeric substrates, and activation of this hydrolysis by various interfaces (Chapus et al., 1976) can be independently studied. Therefore, it seems especially relevant to investigate the influence of a particular residue modification upon these discrete reactions, which are believed to be part of the overall catalysis observed with emulsified substrates.

Materials and Methods

Chemicals. Diethyl *p*-nitrophenyl phosphate (E 600) was purchased from Sigma (St Louis, Mo.). All the other products were obtained from Fluka (Zurich, Switzerland) and were of the best available grade.

Lipase Derivatives. Native lipase was prepared from pig pancreas according to Verger et al. (1969). [^{3}H]Acetyl lipase was prepared as described in the preceding paper (Chapus et al. 1976). 5N-lipase was prepared according to Dufour et al. (1973) by condensation, at pH 5.0, of norleucine methyl ester with lipase in the presence of 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide *p*-toluenesulfonate. DP-lipase was prepared by the method described by Maylié et al. (1972), slightly modified as follows. Lipase solution (about 3 mg/ml) in 0.1 M sodium acetate buffer, at pH 6.0, containing 0.1 M NaCl, 50 mM $CaCl_2$, and 0.3% bovie bile salts, was incubated at room temperature with diethyl *p*-nitrophenyl phosphate (final concentration, 4.6 mM). At the end of the incubation (5 h), lipase activity towards emulsified tributyrin had almost completely disappeared (93–99% inhibition) and 1 mol of *p*-nitrophenol/mol of inhibited enzyme could be measured (Dufour et al., 1973). The modified lipase was then filtered through a (2 × 20 cm) Sephadex G-25 column equilibrated and eluted with a 0.1 M Tris-HCl buffer at pH 7.5. CE-lipase was prepared by reaction with diethyl pyrocarbonate. Lipase (~4 mg/ml), in a 0.1 M phosphate buffer at pH 6.0, was incubated at room temperature with diethyl pyrocarbonate in ethanol solution (final concentration, 3 mM) according to Huc et al. (1971). The formation of carbethoxyhistidine was spectrophotometrically followed at 242 nm. The number of modified histidine residues was obtained by using a molar extinction coefficient of 3200 at 242 nm for carbethoxyhistidine (Ovadi et al., 1967). The enzymatic activity was simultaneously measured on aliquots of the reaction mixture. The reaction completely stopped after 15 min of incubation, due to the rapid hydrolysis of the reagent. In consequence, every 15 min, 3 μmol of diethyl pyrocarbonate was added to 1 ml of the reaction mixture until complete inhibition of the enzyme

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[1] Abbreviations used are: PNPA, *p*-nitrophenyl acetate; E 600, diethyl *p*-nitrophenyl phosphate; 5N-lipase, lipase modified by the addition of five residues of norleucine methyl ester on five carboxyl groups; DP-lipase, diethyl phosphoryl lipase; CE-lipase, lipase modified by carboxymethylation of three histidine residues.

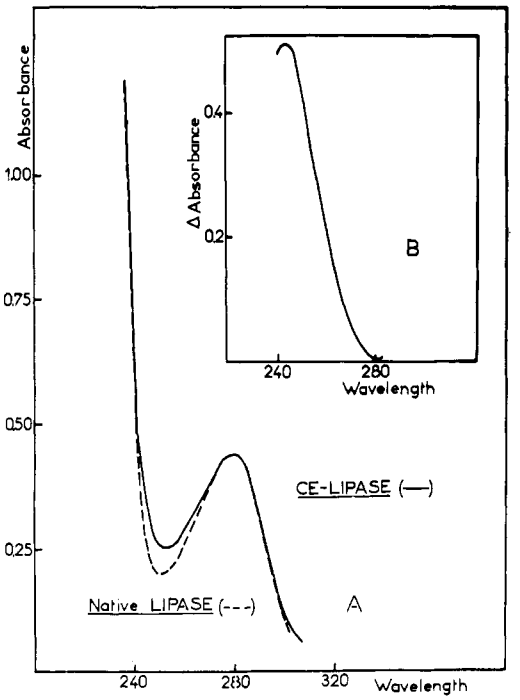

FIGURE 1: Uv spectra of native and diethyl pyrocarbonate modified lipase. (A) Uv spectra of native and modified lipase at identical concentrations (6.6 μM) in a 0.1 M phosphate buffer at pH 6.0. (B) Difference uv spectrum of modified lipase against native lipase (enzyme concentration 30 μM). In these experiments, modified lipase was 80% inhibited. Wavelength in nm.

was achieved. The modified lipase was then filtered through a (1 × 30 cm) Sephadex G-25 column, equilibrated and eluted with a 0.1 M Tris-HCl buffer at pH 7.5.

Lipase Activity Measurements. Activity on emulsified tributyrin was routinely measured at pH 8.0 and 25 °C with 0.5 ml of tributyrin in 15 ml of 0.1 M NaCl (Sémériva et al., 1971). Activity on dissolved substrates was spectrophotometrically measured as previously described (Chapus et al., 1976).

Adsorption of Lipase to Siliconized Glass Beads. The adsorption of lipase on siliconized glass beads was measured as already described (Chapus et al., 1975).

pH Influence on the Reaction of Lipase with Diethyl p-Nitrophenyl Phosphate (E 600). Lipase (15 μM) was reacted in the presence of colipase (35 μM) with E 600 (4.4 mM) in 4 mM taurodeoxycholate and various buffers containing 50 mM $CaCl_2$. The buffers (0.1 M) were respectively: sodium acetate at pH 5.3, 2-(*N*-morpholino)ethanesulfonic acid at pH 5.7, 6.25, and 6.7, and Tris-HCl at pH 7.1. All the buffers were supplemented with NaCl to obtain a final ionic strength of 0.2 M.

Results

Modification of Lipase by Diethyl Pyrocarbonate. The enzyme modified with diethyl pyrocarbonate showed a difference uv spectrum at 242 nm (Figure 1) characteristic of carbethoxyhistidine (Huc et al., 1971). Because of the diethyl pyrocarbonate unstability, the method used to relate the inactivation to the chemical modification was that developed by Tsou-Chen-Lu (1962) and applied to pepsin by Paterson and Knowles (1972). It consists in plotting the remaining activity in function of the number of modified residues. The relation describing the modification is:

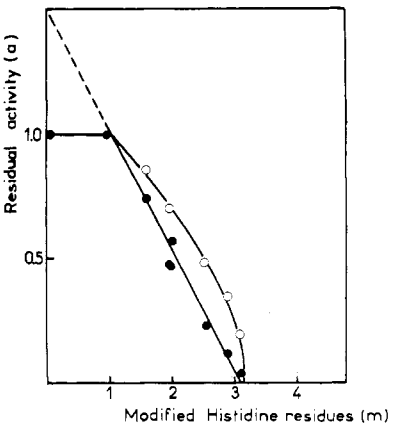

FIGURE 2: Modification of lipase by diethyl pyrocarbonate. The remaining activity (a) to the $1/i$ power was plotted against the number of modified histidine residues (m) according to Tsou-Chen-Lu (1962). (●-●) When $i = 1$; (○-○) when $i = 2$.

$$a^{1/i} = \frac{p + s - m}{2}$$

when $m > s$ and $a^{1/i} = 1$ when $m < s$. In which s is the number of rapidly modified residues with no loss of activity, p is the number of groups reacting slower and at the same rate, among which i is essential, m is the number of modified groups, and a is the remaining activity. The value of i, the number of essential groups, may be obtained from the linearity of the plot $a^{1/i}$ vs. m, when $m > s$.

Figure 2 shows that one histidine residue is rapidly modified without any loss of enzymatic activity on emulsified tributyrin, as already observed in the case of photooxidation (Sémériva et al., 1971), whereas two other residues reacting at the same rate are more slowly modified with a concomitant loss of activity. The results plotted with $i = 1$ and $i = 2$ show that only one of the two "slow" modified histidines is essential to the activity.

It seems that no tyrosine residues are modified by diethyl pyrocarbonate, since there was no change in absorbance at 280 nm. Thiol groups were not modified either by this reagent. Furthermore, the inactivation could be completely reversed at pH 8.0 (50% reactivation in 16 h) or by 0.5 M hydroxylamine at pH 7.5 (50% reactivation in 30 min). This latter result excludes the involvement of amino groups in the loss of activity (Melchior and Farhney, 1970) and is consistent with the relationship existing between inactivation and histidine modification.

Finally, the disappearance of the "burst" obtained with PNPA and the decrease of the steady-state rate hydrolysis of this substrate exactly parallel the loss of activity on emulsified tributyrin (Table I). This result shows that the essential histidine residue modified by diethyl pyrocarbonate is very important for emulsified substrates and monomeric PNPA hydrolysis as well.

Properties of Modified Lipases. Different modified lipases have been tested on emulsified triolein and tributyrin, and on monomeric solutions of PNPA and triacetin. On the other hand, the activation of PNPA hydrolysis, brought about by siliconized glass beads, has also been measured.

Whereas all three forms completely lost their activity on emulsified substrates and their ability to be activated by interfaces, only CE-lipase also lost its activity on monomeric-soluble substrates. By contrast, DP-lipase and 5N-lipase have kept the integrality of their activity on soluble substrates.

TABLE I: Effect of Modification of Histidine Residues on Emulsified Tributyrin and Monomeric-Soluble PNPA Hydrolysis.[a]

	Steady-State Rate of Emulsified Tributyrin Hydrolysis (arbitrary units)	"Burst" on PNPA (mol of PNP Released/mol of Lipase)	Steady-State Rate of PNPA Hydrolysis (arbitrary units)
Native lipase	1	1	1
Diethyl pyrocarbonate modified lipase			
2 His modified	0.57	0.53	0.62
2.6 His modified	0.22	0.22	0.31

[a] Inactivation was followed by measuring the remaining lipase activity on emulsified tributyrin in function of incubation time with E 600. First-order kinetics were obtained up to 80–90% inhibition. For experimental conditions, see Methods.

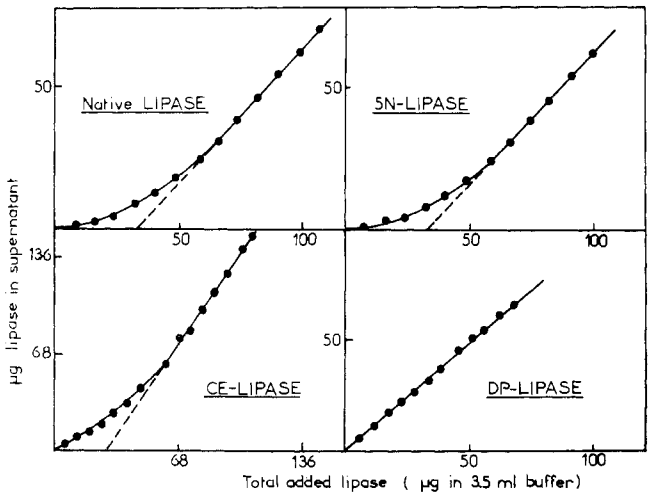

FIGURE 3: Adsorption of modified lipases on siliconized glass beads. After settling down of the beads, protein concentration in the supernatant was determined by optical density at 220 nm and plotted against total enzyme concentration added.

Moreover, it has been demonstrated that the kinetic constants of DP-lipase for PNPA hydrolysis were very close to that observed with native lipase.

The ability for the modified lipases to be adsorbed to siliconized glass beads interface has also been investigated. Modified lipases seem to be less resistant to denaturation by adsorption to interfaces. Consequently, all the adsorption experiments were carried out in presence of a 2 molar excess of colipase. Figure 3 shows that 5N-lipase and CE-lipase have normal adsorption curves when compared to native lipase, whereas DP-lipase is unable to be adsorbed to this interface. The loss of activity of DP-lipase on emulsified substrates must be, thus, attributed to its inability to be adsorbed on interfaces.

The lack of participation of the essential serine residue to the catalytic site per se was further demonstrated by a study of the pH influence on the inhibition rate of lipase by E 600.

As shown in Table II, this inhibition does not obey the same

TABLE II: Influence of pH on the Inhibition of Lipase by E. 600.

pH	First-Order Rate Constant of Inhibition (min^{-1})	V_{max} (μmol min^{-1} mg^{-1}) on emulsified tributyrin[a]
5.3	0.3	1720
5.7	0.28	4000
6.25	0.26	6900
6.7	0.23	8000
7.1	0.20	8400

[a] From Sémériva et al., 1971.

pH dependency as the activity. The rate of inactivation was roughly constant with respect to pH, whereas lipase activity is controlled by an ionizing group of pK 5.8, which must be unprotonated for the enzyme to be fully active (Sémériva et al., 1971).

Discussion

In the case of pancreatic lipase, chemical modifications can a priori be delineated according to their effect upon the three discrete reactions of the overall catalysis, which can be independently studied. (1) Modifications which change the interfacial adsorption properties of the enzyme and, thus, have a direct consequence on the first step of catalysis. In this case, no activity on emulsified substrates may be expected, whereas a full activity on soluble substrates may still remain. An illustration of the importance of the adsorption step has recently been demonstrated (Chapus et al., 1975). (2) Modifications which, although they apparently do not affect the adsorption step, produce an enzyme unable to be adsorbed in the right manner to be fully active, and then, consequently, impair the interfacial activation. This must lead to a loss of activity on emulsified substrates and a loss of activability by interfaces. The modified forms can, however, display a full activity on soluble substrates and have apparently normal adsorption curves. (3) Modifications which abolish all types of activity on soluble and emulsified substrates as well. Adsorption curves may or may not be identical to that observed with native lipase. The essential residue is probably at (or very close to) the active center.

It seems that these three types of modification have been obtained. The modification of one essential histidine residue in CE-lipase led to the complete loss of activity on both emulsified and dissolved monomeric substrates. This result suggests that this essential residue is situated at (or very close to) the active center and confirms the hypothesis that the same catalytic site is responsible for the hydrolysis of both types of substrates (Chapus et al., 1976).

By contrast, 5N-lipase has lost its activity on emulsified substrates and its activability by interfaces, but has preserved its ability to hydrolyze soluble substrates. Adsorption curves show an apparently normal behavior of these modified enzymes with respect to the first step of catalysis. The essential carboxyl group is, thus, proposed to play an important role in the interfacial activation.

The most interesting results were obtained with DP-lipase. This form has lost its activity on emulsified substrates and also its activability by interfaces, but is fully active on soluble substrates. The fact that DP-lipase is still able to hydrolyze soluble PNPA deserves a special comment. This result shows that PNPA and diethyl p-nitrophenyl phosphate, despite the similarity of their chemical structure, do not react with the same site on the enzyme. Furthermore, [^{3}H]acetyl lipase can

still be prepared from DP-lipase without an exchange of the diethyl group being observed. It cannot be excluded, of course, that diethyl *p*-nitrophenyl phosphate primarily reacts with the same acylable residue but is transferred afterwards to a serine residue close to the former one. Such transfers have been already mentioned (Delamare et al., 1972). Nevertheless, the experimental results challenge the idea that the diethyl phosphoryl group was fixed at the acylable center of lipase (Maylié et al., 1972). This hypothesis was previously suggested by analogy with serine esterases which possess an acylable serine residue, which can be modified by diethyl *p*-nitrophenyl phosphate (Hartley and Kilby, 1954). This hypothesis is, also, very unlikely regarding that lipase inactivation by E 600 and lipase activity on emulsified substrates are not controlled by the same ionizing group. These observations suggest that the essential serine residue is rather involved in the adsorption of lipase to interfaces. This was supported herein by the fact that DP-lipase was unable to adsorb on siliconized glass beads interface.

Finally, it is noteworthy that the modification of only one residue leads to a form unable to be adsorbed at interfaces. The interfacial recognition site must be, thus, very specific, as it was, recently, very well demonstrated in the case of another lipolytic enzyme, the pancreatic phospholipase A_2 (Slotboom and de Haas, 1975). The comparison of adsorption to interfaces of lipase and other proteins, and also of lipase in presence or in absence of bile salts and (or) colipase, had already led to the same conclusion (Chapus et al., 1975).

A tentative mechanism for lipase can be proposed. Lipase is supposed to possess an esteratic catalytic site preformed in solution, although acting with a low efficiency. Hydrolysis of soluble substrates occurs via an acyl enzyme mechanism in which a histidine residue and a yet unknown acylable residue (X) play essential roles, in a similar way as for other esterases.

In addition to this catalytic site, lipase possesses a topographically distinct site that is responsible for the specific interaction and activation by interfaces, which would correspond to the interfacial recognition site of phospholipase A_2 (van Dam-Mieras et al., 1975). In presence of an interface, lipase acquires a different conformation, which is more efficient than the one occurring in solution. The adsorption step is controlled by a serine residue, which is essential for the recognition of the interface. A carboxyl group is also of importance in adjusting or stabilizing the best active conformation of lipase. The consequence of this conformational change is to accelerate deacylation and acylation rates. Other factors due to the adsorption of substrate can also be of importance in these steps. The histidine and X residues are supposed to play here the same role as in the absence of interface. Since the desorption rate of lipase is slow (Brockman et al., 1973) and the lateral diffusion of a protein at interface is very fast (Lee et al., 1973), it is suggested that lipase laterally translates at the interface to find another substrate molecule.

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