another study of NDP-kinase isolated from calf thymus, Nakamura and Sugino (1966) separated two peaks of NDP-kinase activity by chromatography on DEAE-cellulose at pH 7.5. These fractions could not be distinguished from each other with respect to their specificity toward nucleoside triphosphates, optimal pH, and metal requirements, but sucrose density gradient centrifugation suggested the presence of at least two forms of enzyme with different sedimentation rates. The recent studies of Edlund *et al.* suggest the possible occurrence of isozymes of NDP-kinase in baker's yeast since phosphocellulose chromatography separated one main activity peak preceded by a small one. In some preparations the two activity peaks were of nearly the same size. However, to our knowledge there has not been a clear-cut demonstration of isozymes of NDP-kinase prior to the present report.

Acknowledgment

We thank Dr. Park Gerald and his colleagues at the Boston Children's Hospital for assistance in developing the agarose electrophoresis method and the New England Enzyme Center at Tufts University for preparing partially purified NDP-kinase from pooled blood.

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On the Probable Involvement of a Histidine Residue in the Active Site of Pancreatic Lipase*

M. Sémériva, C. Dufour, and P. Desnuelle†

ABSTRACT: In the course of this work, two lines of evidence were obtained consistent with the view that a histidine residue participates in the active site of porcine pancreatic lipase. (1) The maximal rate of lipase-catalyzed hydrolysis of tributyrin emulsions is under the control of an ionizable group of pK = 5.8 which must be unprotonated. The parallel variation of K_m and V_m in this pH range has been interpreted as showing that K_m is not a true equilibrium constant in the case of lipase. The acylation of the enzyme, probably occurring at an oil–water interface, is likely to be slower than deacylation. (2) Lipase is rapidly and completely inactivated according to a first-order reaction by photooxidation in the presence of a dye sensitizer. Tryptophan, cysteine, methionine, and histidine were found to be modified in photooxidized lipase. Measured oxidation rates were quite different according to the type of residue and also to the "reactivity" of the various residues of a given type in the protein molecule. The simplest correlation between inactivation and photooxidation of a single residue was found for the second most reactive histidine. However, other correlations are possible as in most photooxidation assays. Most of them could be ruled out with the aid of suitable techniques.

A number of investigations have been carried out in recent years on the mode of action of pancreatic lipase and on the minimum structural requirements of its substrates. In contrast, few results have so far been obtained concerning the amino acid residues involved in its catalytic and binding sites. Nevertheless, lipase is a special kind of esterase characterized by its unique ability to act with an unusually high speed on emulsified or micellar substrates (Sarda and Desnuelle, 1958;

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Entressangles and Desnuelle, 1968). It is of interest, therefore, to know which structural arrangement in the enzyme molecule can be held as responsible for this ability.

A first approach to the problem was recently made in this laboratory when the reaction of porcine pancreatic lipase with organophosphates was investigated. Diisopropylphosphofluoridate, a potent inhibitor of esterases, was shown to bind, when used in high concentrations, quite selectively to one tyrosine residue of the enzyme molecule, but to have no effect on its activity (Maylié *et al.*, 1969). Conversely, diethyl *p*-nitrophenyl phosphate in the presence of bile salts was found to inhibit lipase by a stoichiometric reaction probably involving an essential serine residue (Maylié *et al.*, 1969, and unpublished experiments).

Porcine pancreatic lipase was also observed to contain two SH groups with different reactivities (Verger *et al.*, 1970). However, lipase is certainly not a sulfhydryl enzyme since a substantial part of its activity is retained after substitution of both groups by a phenylmercuric radical (R. Verger *et al.*, manuscript in preparation). A tentative hypothesis is that one of the groups, or both, are situated in a region of the molecule playing an important role for the specific binding of the enzyme to hydrophobic interfaces (R. Verger *et al.*, manuscript in preparation).

In the present paper, two lines of evidence are presented in favor of the possible participation of an histidine residue in the active site of porcine pancreatic lipase. The first was obtained by a study of the action of protons on activity, which appeared to be under the control of a group with a pK of 5.8. The second was derived from photooxidation assays. In spite of the known lack of selectivity of this latter technique resulting in the modification of quite a number of residues, an excellent correlation could be established between the rate of enzyme inactivation and the rate of oxidation of a histidine residue. The obvious objection that a number of other correlations existed with the photooxidation of several other residues could be largely overcome with the aid of suitable techniques.

Materials and Methods

Preparation of Lipase. Lipase was purified from fully defatted porcine pancreas extracts according to the technique of Verger *et al.* (1969). The mixture of the two enzymes L_A and L_B emerging from the Sephadex G-100 column was used throughout this work.

Determination of the Kinetic Parameters of Lipase Using a Tributyrin Emulsion. For kinetic investigations, it was found necessary to modify the test currently used for the determination of lipase activity and to employ tributyrin as substrate. Tributyrin has over long-chain triglycerides several distinct advantages. (a) It emulsifies spontaneously in dilute NaCl solutions under moderate agitation, so that no emulsifier is required. Emulsifiers may be expected to accumulate at oil–water interfaces and to interfere with lipase activity. (b) Butyric acid liberated during hydrolysis is water soluble so that linear kinetics are obtained for an appreciable period of time, even in the absence of bile salts. Bile salts are quite embarrassing since their optimal concentration varies (Maylié *et al.*, 1971) with the degree of purity of lipase and the composition of the test mixture. Moreover, they were shown to shift the optimum pH of lipase (Borgström, 1954) and consequently to modify the pK's of its essential ionizable groups. (c) Butyric acid has a lower pK than long-chain fatty acids with the consequence that lipolysis can be followed titrimetrically without undue corrections at pH values as low as 5.0. Conversely, commercial tributyrin must be carefully purified and, since no preformed emulsion is used, the reproducibility of the emulsification of this triglyceride is critical.

Commercial tributyrin (Fluka, Zurich) was purified by passage through a 5% hydrated Florisil column equilibrated and eluted with a hexane–diethyl oxide mixture (80:20, v/v). The desired amounts of this substrate were weighted in carefully calibrated pH-Stat vessels and emulsified in 15 ml of 100 mM NaCl by a 5–10-min stirring at 1100 rpm. The liberation of butyric acid at the selected pH under a stream of nitrogen was recorded at 25° as a function of time with the aid of a Radiometer pH-Stat Model TTT 1a charged with 20 mM NaOH. Kinetic curves were linear for at least 2–3 min and reproducibility was found to be within ±3%. The pH-Stat response was strictly proportional to the amount of added lipase in a range extending from 0.1 to 4 enzyme units.

Moreover, strict linearity of the $1/v$ plots *vs.* $1/(S)$ over a large substrate concentration range facilitated the determination of the two kinetic parameters (K_m and V_m) of lipase. However, K_m values were not expressed, as they should be (Benzonana and Desnuelle, 1965), in interface concentrations (square meters of interface in 1 l. of emulsion), but more simply in arbitrary units proportional to the weight of tributyrin added to a known volume of NaCl solution. This mode of expression is correct provided that the emulsions are well standardized.

Photooxidation Technique. Since the reaction rate and reproducibility were of importance in this technique, experimental conditions were carefully adjusted. The general procedure was this described by Ray and Koshland (1962). The reaction vessel (10 ml) was plunged in a thermostated glass water bath and a 300-W spotlight (PAR 56/NSP General Electric) was fixed below this bath at exactly 30 cm from the bottom of the vessel. Vessel and spotlight were mounted on a horizontal shaker so adjusted as to give 60 strokes of 4.5-cm length per min. A temperature of 13.5° was maintained in the bath by the use of a stream of cold water and a thermoregulator unit.

Two milliliters of 50 mM Tris-HCl buffer (pH 8.0) containing 8 mg of lipase and 0.01% methylene blue was placed in the vessel flushed with pure oxygen. The rate of inactivation was independent of lipase concentration in the range 0.5–5.0 mg/ml. After each illumination period, aliquots were removed and kept at 0° in the dark before assay.

The photooxidation effect was found to vary with the volume of the illuminated solution. A correction factor must therefore be applied after each removal of aliquots. Figure 1 indicates the values of this factor derived from experiments performed on aqueous solutions of free histidine in the presence of 0.01% methylene blue.

Determination of Lipase Concentration in the Photooxidized Solutions. The dye-sensitizer methylene blue was first removed by filtration under dim light of the illuminated solutions through a 1.1 × 12 cm Sephadex G-25 column equilibrated with a 50 mM Tris-HCl buffer (pH 8.0).

From the outset of the photooxidation, lipase concentration could not be determined by direct spectrophotometry at 280 nm, because of the progressive degradation of tryptophan. This concentration, therefore, was measured according to Lowry *et al.* (1951).

Amino Acid Analysis. The photooxidized lipase was freed from the dye sensitizer either by Sephadex filtration or by precipitation in 10% trichloroacetic acid. In the latter case, the mixture was kept in ice for 2 hr and the precipitate was washed twice with 1 ml of 5% trichloroacetic acid and three

times with 1 ml of 95% ethanol. The percentage of oxidized residues was derived from a number of analyses by the automatic procedure of Spackman *et al.* (1958) after a 24-hr hydrolysis in triple-distilled HCl or a 36-hr hydrolysis in 1 ml of 1 N NaOH. Values for the modified residues were normalized to the Asp, Glu, Pro, Ala, Ile, Leu, and Lys peaks, since the corresponding amino acids were expected to be unaffected by photooxidation and found to be obtained with the highest accuracy and reproducibility. Tryptophan was determined by the spectrophotometric method of Spies and Chambers (1949) and cystine as cysteic acid after performic oxidation of the protein. The reactive and nonreactive SH groups (SH_I and SH_{II}) (Verger *et al.*, 1970) were estimated according to Ellman (1959) before and after denaturation of the protein by 0.3% sodium dodecyl sulfate.

Methionine sulfoxide arising from the photooxidation of methionine residues could be expected to reverse to methionine during acid hydrolysis (Ray and Koshland, 1962). For this reason, the percentage of photooxidized methionine was calculated from the results obtained after alkaline hydrolysis.

Regeneration of Methionine from the Sulfoxide. Photooxidized lipase was incubated at pH 9.0 with 1 or 10 mM mercaptoethanol for 48 hr or with a 30-fold molar excess of dithiothreitol for 30 min. It has been ascertained that the methionine sulfoxide arising during photooxidation was quantitatively converted into methionine at the end of this treatment. Moreover, no detectable traces of methionine sulfone could be found in photooxidized lipase.

Lipase Derivatives. S-Diphenylmercuri-lipase was prepared in this laboratory by Dr. R. Verger by action of phenylmercuric chloride on the native enzyme (R. Verger *et al.*, manuscript in preparation). Another sample of lipase was treated in 10% acetone with 2-hydroxy-5-nitrobenzyl bromide according to Koshland *et al.* (1964). The number of modified tryptophan residues was evaluated spectrophotometrically at 410 nm in an alkaline solution.

Results

pH Dependence of the Kinetic Parameters (K_m and V_m) of Lipase-Catalyzed Reaction. Reciprocal Lineweaver–Burk plots $1/v$ *vs.* 1/(S) for the lipase-catalyzed hydrolysis of tributyrin emulsions were established for a number of pH values between 5.0 and 10.5. No sizeable inactivation of lipase was observed at any pH during the time required for the measurement. Results obtained below pH 6.0 were corrected to compensate the partial ionization of butyric acid. The variations of V_m and K_m as a function of pH are indicated by Figures 2 and 3, respectively.

Figure 2 shows that the experimental values obtained for V_m at various pH's fit well with a bell-shaped theoretical curve drawn under the assumption that this parameter is under the control of two groups with pK's of 5.8 and 10.1, respectively. For V_m to be maximal, the pK = 5.8 group must be unprotonated and the pK = 10.1 group, protonated. The enthalpy of ionization of the pK = 5.8 group was calculated to be 5000 cal/mole.

The plot of K_m *vs.* pH (Figure 3) also results in a bell-shaped curve with a rather sharp maximum for pH 7. In the acidic range, the participation of a group with a pK of 5.8 is again indicated. On the alkaline side, another group with a pK of 8.2 is apparently involved. However, the experimental points deviate noticeably in this region from the theoretical curve corresponding to a single ionizing group of pK = 8.2. No conclusion, therefore, can be drawn.

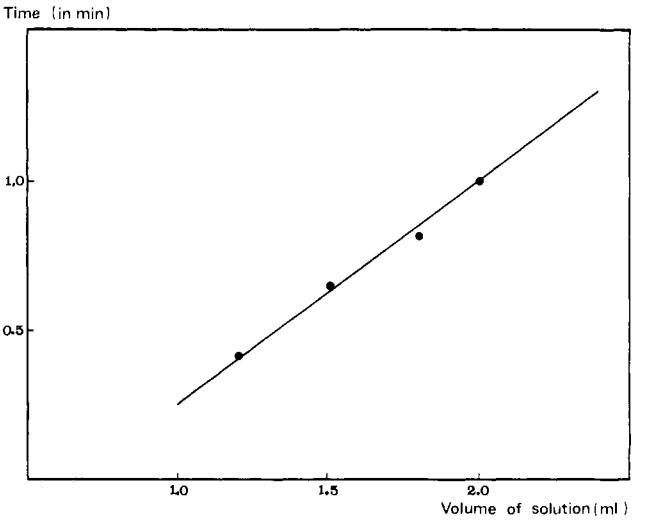

FIGURE 1: Photooxidative degradation rate of histidine as a function of the volume of solution. Histidine (1 mM) was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 0.01% methylene blue. The ordinate is the time required to achieve the same extent of photooxidation of histidine solutions that was observed after a 1-min reaction time with a 2-ml reaction volume. The histidine remaining after photooxidation was determined with Pauly's reagent.

Photooxidation of Lipase. INACTIVATION. Lipase inactivation by photooxidation as measured by the decrease of tributyrin hydrolysis rate was found to be a very fast first-order reaction up to 90% (Figure 4). The rate constant of this reaction (k_a) was 0.160 min^{-1}. The inactivation could be observed to be induced by a drop of V_m whereas K_m remained unchanged.

EFFECT OF TRYPTOPHAN RESIDUES. The kinetic interpretation of this and the following experiments was made according to Ray and Koshland (1961). The shape of the semilogarithmic plot reproduced in Figure 5 suggests that two classes of tryptophan residues exist in native lipase. The first are oxidized very fast (k_1 = 0.49 min^{-1}) and the other more slowly (k_2 = 0.015 min^{-1}). It can be calculated that two tryptophan residues (22%) are reactive while five (78%) are unreactive toward photooxidation.

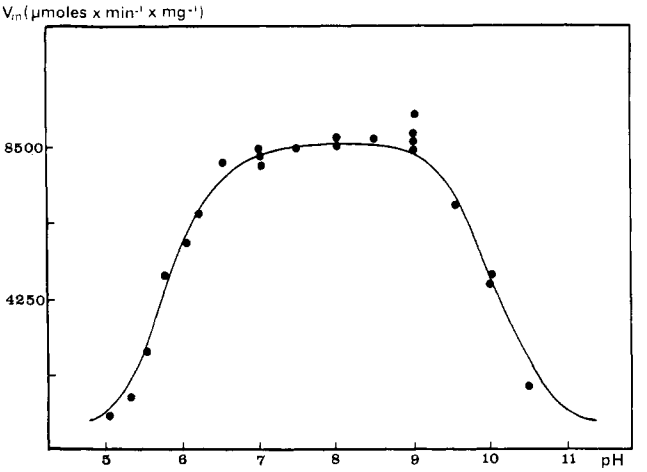

FIGURE 2: pH dependence of V_m. Experimental values are represented by filled circles. The solid line is a theoretical curve calculated using values of pK_1 = 5.8 and pK_2 = 10.1. Lipase activity was measured on tributyrin emulsions at 25° (see Methods).

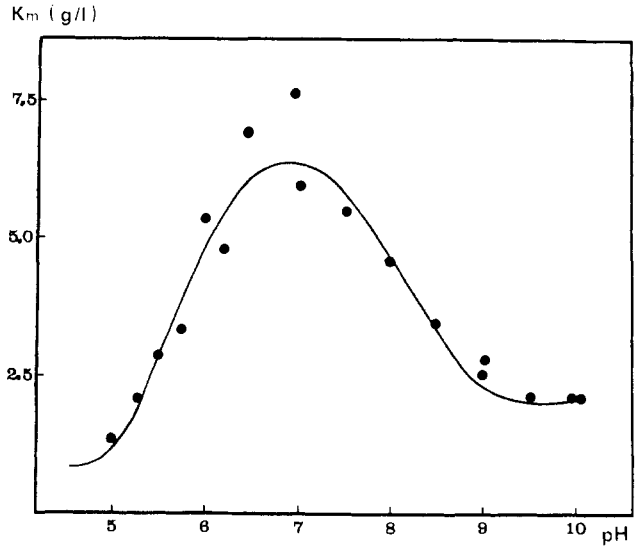

FIGURE 3: pH dependence of K_m. Experimental values are represented by filled circles. The solid line is a theoretical curve calculated using values of $pK_1 = 5.8$ and $pK_2 = 8.2$. See Methods for the experimental conditions and the mode of expression of K_m (ordinates).

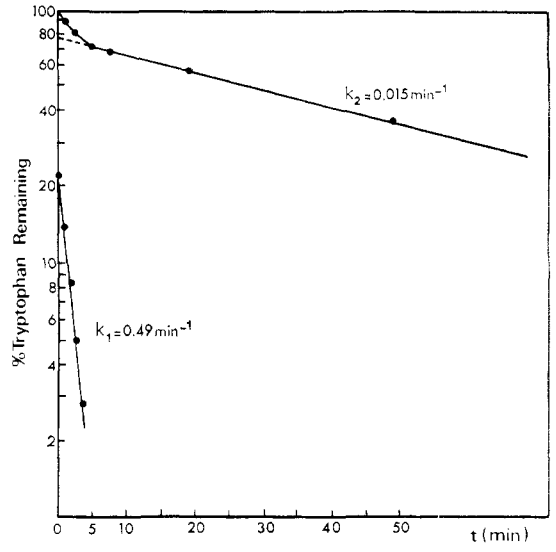

FIGURE 5: Photooxidation of tryptophan residues. k_1 and k_2 designate the photooxidation rate constant for, respectively, the reactive and unreactive tryptophan residues.

EFFECT ON CYSTEINE RESIDUES. Lipase is already known to possess two SH groups. One (SH_I) is readily "accessible" in the native state to 5,5′-dithiobis(2-nitrobenzoic) acid and to other polar sulfhydryl reagents while the other (SH_{II}) is "inaccessible" unless the enzyme is denatured. The case of these groups is of special interest since condensation of the native or denatured enzyme with the above reagent permits a direct quantitation of each kind of residues whose photooxidation can therefore be independently followed.

Figure 6 demonstrates that the SH_I and SH_{II} groups are actually photooxidized at two widely different rates ($k_1 = 0.008$ min^{-1}; $k_2 = 0.12$ min^{-1}). However, the SH_{II} group which is "inaccessible" to polar sulfhydryl reagents (see above), is the one to be most rapidly photooxidized. Photooxidation of the "accessible" group is much more sluggish.

EFFECT ON METHIONINE RESIDUES. Figure 7 shows that two different reactivities can also be discerned among methionine residues. One is photooxidized relatively fast ($k_1 = 0.290$ min^{-1}) whereas the other three react quite slowly ($k_2 = 0.042$ min^{-1}).

EFFECT ON HISTIDINE RESIDUES. The photooxidation of histidine was investigated in much detail since a direct participation of an imidazole in the active site of lipase was already suggested by the titration assays reported above. Interpretation of the data was somewhat complicated by the relatively high histidine content of the enzyme (10 residues/mole).

At first sight, Figure 8 seems to indicate that two classes of histidine residues shall also be discerned in lipase molecule,

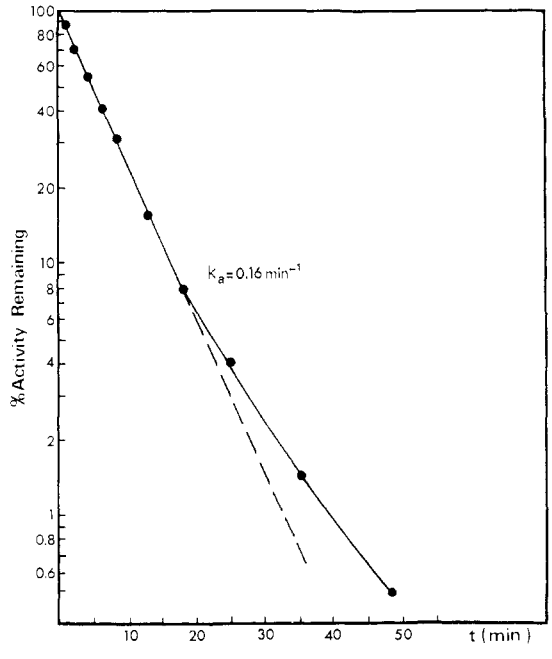

FIGURE 4: Inactivation of lipase by photooxidation. The lipase activity remaining after photooxidation was measured at 25° and pH 8.0 against 0.5 g of tributyrin emulsified in 15 ml of 100 mM NaCl.

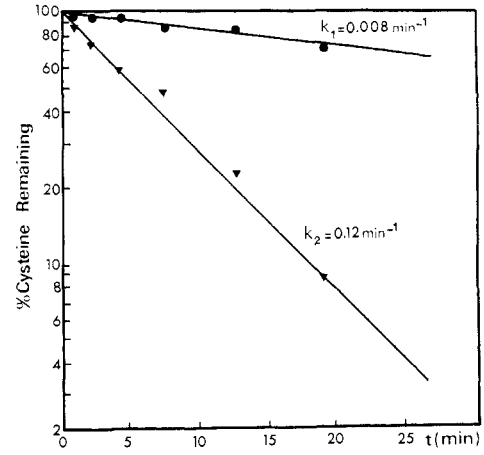

FIGURE 6: Photooxidation of cysteine residues. Filled circles and triangles correspond, respectively, to the SH_I and SH_{II} groups present in porcine pancreatic lipase.

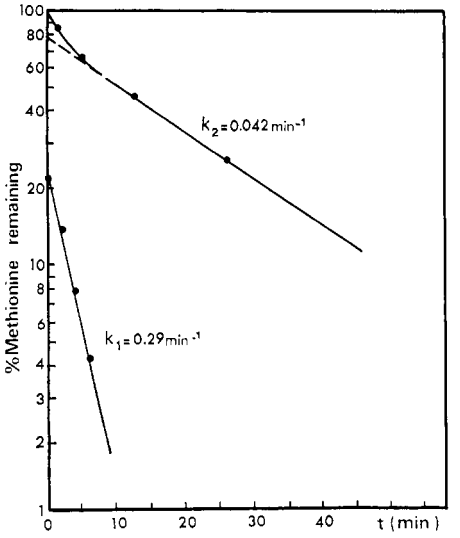

FIGURE 7: Photooxidation of methionine residues. One out of the four methionine residues of porcine lipase is photooxidized rapidly ($k_1 = 0.220$). The other three react more slowly ($k_2 = 0.042$).

one including eight residues with a first-order photooxidation rate constant of 0.021 min^{-1} and the other including two residues with a much higher constant. However, a closer examination of the data shows that the photooxidation of the latter two residues does not strictly follow a first-order kinetic. Results obtained in a number of assays were consistent with the view that one residue is oxidized very fast (first-order rate constant, 0.590 min^{-1}) while a constant equal to 0.150 min^{-1} can be attributed to the second. However, it is clear that this subdivision of the fast-reacting histidines needs confirmation by an independent approach.

No significant loss of residues other than tryptophan, cysteine, methionine, and histidine could be detected by analysis of photooxidized lipase samples.

Discussion

Effect of pH on the Kinetic Parameters V_m and K_m. It can be concluded from the bell-shaped curve in Figure 2 that the V_m of lipase-catalyzed tributyrin hydrolysis is under the control of two ionizable groups in the enzyme. For V_m to reach its maximal value, the first group with a pK of 5.8 must be unprotonated while the second with a pK of 10.1 must be protonated. Imidazole is the single grouping in proteins whose ionization state strongly varies around pH 5.8. This latter value is distinctly lower than the pK of free histidine. But, an even stronger downward shift is observed for one of the histidine residues involved in the active site of ribonuclease (Herries *et al.*, 1962) (pK = 5.22). An additional argument in favor of histidine being involved in lipase activity is the value of 5000 cal/mole calculated for the ionization enthalpy of the pK = 5.8 group. This value is fully consistent with the deionization of an imidazolium ion (Cohn and Edsall, 1943). In contrast, the identification of the second group with a pK of 10.1 is not possible at the present stage of our investigations, because of the variety of protein groups ionizing in this pH range.

The plot of K_m *vs.* pH (Figure 3) also results in a bell-shaped curve suggesting the participation in the acidic range of a group with a pK of 5.8. Although the transitory appearance of an acyl derivative of lipase has not been experimentally

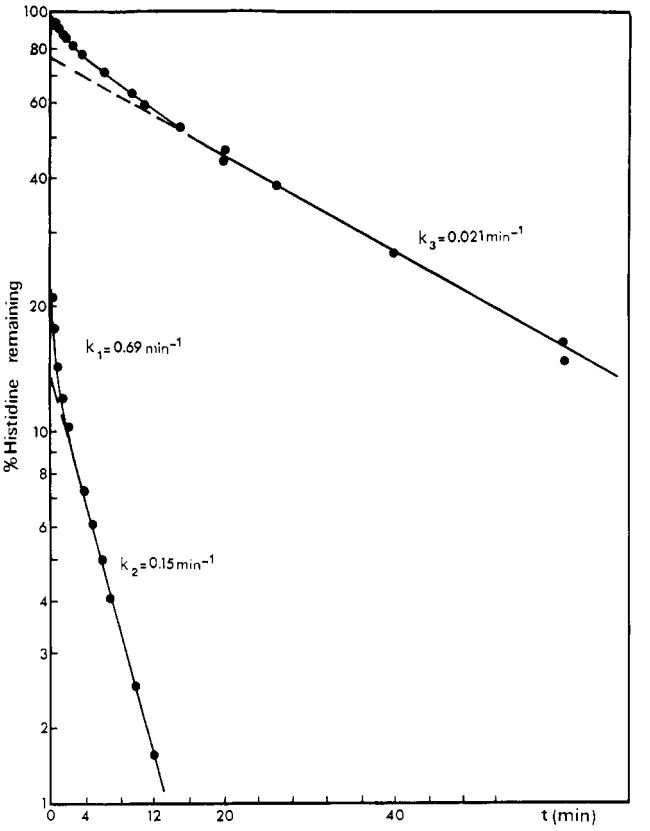

FIGURE 8: Photooxidation of histidine residues. The three constants k_1, k_2, and k_3 correspond to three subclasses of histidine residues with decreasing reactivity.

proved, the simplest model for lipolysis seems, by analogy with esterolysis, to be

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

which leads to $K_m = k_3(k_{-1} + k_2)/k_1(k_2 + k_3)$. Since the rate of the lipase-catalyzed hydrolysis of ester substrates has been shown to be much more affected by the nature of the alcohol moiety than by the acyl radical (Brockerhoff, 1968), the acylation of the enzyme at hydrophobic interfaces appears to be slower than the further decomposition of acyl-lipase, with the consequence that $k_2 \ll k_3$, and $K_m = k_{-1} + k_2/k_1$.

Moreover, the above reported parallel change in V_m and K_m at low pH may be reasonably interpreted as showing that k_{-1} and k_2 are of the same order of magnitude. In conclusion, the K_m of lipase is not likely to be a true equilibrium constant and the following inequality probably holds: $k_3 \gg k_2 \doteq k_{-1}$.

Photooxidation Effect on Lipase Activity. In order to obtain an independent confirmation of the participation of an histidine in lipase activity, the enzyme was photooxidized in the presence of a dye sensitizer. Since photooxidation is not specific for imidazole, but also affects a number of other groupings, it was necessary to demonstrate, as in similar previously investigated cases (Ray and Koshland, 1962; Koshland *et al.*, 1962; Friedrich *et al.*, 1964; Martinez-Carrion *et al.*, 1967) that, among all the observed oxidations, this of histidine correlated best with inactivation.

The first-order rate constants for the photooxidative inactivation of lipase (Figure 4) and for the destruction of trypto-

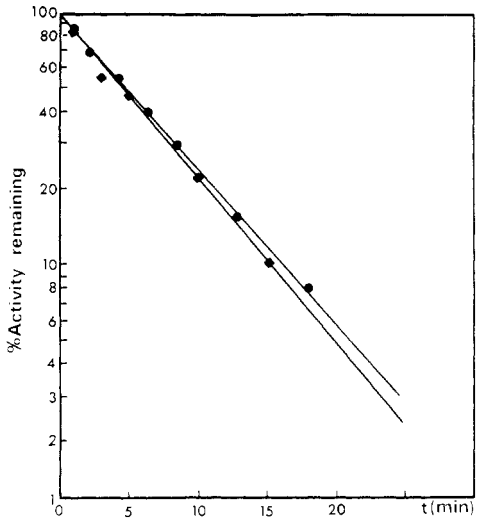

FIGURE 9: Photooxidative inactivation of lipase (circles) and of its *S*-diphenylmercuric derivative (triangles).

phan, cysteine, methionine, and histidine (Figures 5–8) are reproduced in Table I for comparison.

Among the nine oxidation rate constants listed in Table I, three related to the two reactive tryptophans, to the single reactive methionine and to the most reactive histidine are much higher than the inactivation constant. Two possibilities exist for these residues. Either they do not participate at all in lipase activity, or their destruction merely induces a partial inactivation. In contrast, the photooxidation of the second most reactive histidine (rate constant 0.150 min^{-1}) correlates almost exactly with inactivation (rate constant 0.160 min^{-1}) and the assumption can be made that this residue is essential for activity. However, a number of other correlations are obviously possible (Ray and Koshland, 1961) with the photooxidation of several of the less reactive residues such as the remaining five tryptophans, three methionines, eight histidines, and the two cysteines. A closer examination of the case of these residues, therefore, seems to be necessary.

TABLE I: Calculated First-Order Rate Constants.[a]

Reactions	Rate Constants (min^{-1})
Lipase inactivation	0.160
Tryptophan oxidation	
Reactive	0.490
Unreactive	0.015
Methionine oxidation	
Reactive	0.290
Unreactive	0.042
Cysteine oxidation	
SH_I	0.008
SH_{II}	0.120
Histidine oxidation	
Reactive I	0.690
Reactive II	0.150
Unreactive	0.021

[a] The numerical values listed in this table are taken from Figures 4 to 8.

In contrast to earlier claims discussed by Wills (1960), pancreatic lipase has now been definitely proved not to be a sulfhydryl enzyme. A substantial part of its activity is retained after oxidation of the two SH groups by iodine (M. F. Malié, unpublished experiments) or after reaction with phenylmercuric chloride (R. Verger, manuscript in preparation). This is already a quite convincing argument against the possibility that photooxidation of cysteine is responsible for inactivation. Besides, an independent confirmation was obtained by photooxidizing in parallel the enzyme and its *S*-diphenylmercuric derivative. Figure 9 shows that both compounds are inactivated at almost exactly the same rate. Since the sulfhydryls of 6-phosphogluconate dehydrogenase have been demonstrated to be effectively protected against photooxidation by mercuribenzoate (Rippa and Pontremoli, 1968), this experiment rules out any possible contribution of the SH groups.

Moreover, regeneration of methionine from the sulfoxide by incubation of photooxidized lipase with mercaptoethanol or dithiothreitol was not observed to induce any reactivation. It has already been pointed out before that all the methionine sulfoxide residues in photooxidized lipase were converted into methionine by incubation with thiols and that no methionine sulfone was formed during photooxidation. Hence, the modification of methionine is not likely to play an important role in inactivation.

A sample of native lipase was also treated by 2-hydroxy-5-nitrobenzyl bromide, a reagent known for its specific binding to indol rings. Under the assumption that two hydroxynitrobenzyl radicals are attached to each ring (Barman and Koshland, 1967) and using the molar extinction coefficient value given by the authors, it was possible to calculate that two tryptophan residues had reacted in lipase under the conditions employed for incubation. The modification of these residues did not induce any perceptible inactivation. The problem regarding the other tryptophan of the enzyme molecule, however, is still unsolved. Similarly, any possible contribution of the other histidines in the inactivation process cannot for obvious reason be evaluated.

Uncharged imidazols are generally considered to be more sensitive to photooxidative degradation than imidazolium ions (Westhead, 1965). As a consequence, the photooxidation of histidine in proteins and the oxidative inactivation of histidine-dependent enzymes should be expected to be slower in the acidic pH range. In the case of lipase, more than half of the histidine residues oxidized at pH 8.0 were observed to be also oxidized at pH 5.6, whereas the inactivation constant was 20% lower. The possibility for the process to be effectively controlled by pH was not further explored for the reason that lipase contains ten histidine residues with probably quite different p*K*'s.

In conclusion, the simplest interpretation of the data is that the second most reactive histidine residue is likely to participate in the activity of pancreatic lipase against an emulsified substrate. Any important participation of cysteine, of methionine and of at least two tryptophan residues seem to be ruled out by the results given by independent techniques. However, these results do not exclude the still possible participation of the other tryptophans and histidines. A final answer in this respect cannot be given before the discovery for lipase of a bifunctional inhibitor similar to the ones used by Shaw in the case of chymotrypsin (Schoellmann and Shaw, 1963), trypsin (Shaw *et al.*, 1965), and subtilisin (Shaw and Ruscica, 1968).

It should also be emphasized that the invariance of K_m during photooxidation of lipase does not necessarily mean

that the oxidized group(s) exclusively participates in the catalytic site of the enzyme, and not in the binding site. Indeed, the photooxidative inactivation may be assumed to be an "all-or-none" process, each active molecule in the mixture remaining fully active. The validity of this assumption could not be checked because no reagent permitting an active-site titration is yet available in the case of lipase.

In addition, the chemical modification(s) associated with photooxidation inactivation is likely to exert a direct effect on the activity of the enzyme molecule, and not merely on its conformation. No indication of denaturation could be detected in photooxidized lipase. The reaction rate of the SH_I group with 5,5-dithiobis(2-nitrobenzoic) acid was not increased during photooxidation, as it is known to be upon treatment by sodium dodecyl sulfate or urea (Verger *et al.*, 1970). On the other hand, the SH_{II} group remained fully unreactive. A further proof that lipase essentially retained its native conformation during photooxidation was given by the fact that the degradation rate of unreactive methionine, tryptophan, and histidine residues was not appreciably modified at the end of photooxidation assays of very long duration (up to 99.9% inactivation).

A last remark is that the group(s) modified by photooxidation appears to be really "essential" for the activity of lipase, since an almost complete inactivation can be observed at the end of the assays without any detectable signs of denaturation.

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