BBA 66396

ON THE SULFHYDRYL GROUPS OF PORCINE PANCREATIC LIPASE AND THEIR POSSIBLE ROLE IN THE ACTIVITY OF THE ENZYME

R. VERGER, L. SARDA AND P. DESNUELLE
Institut de Chimie Biologique, Université de Provence, Marseille (France)

(Received April 13th, 1971)

SUMMARY

Porcine pancreatic lipase contains two SH groups (SH_I and SH_{II}). The SH_I group is reactive in the native enzyme towards sulfhydryl reagents such as 5,5'-dithiobis(2-nitrobenzoic)acid, *p*-chloromercuribenzoate and *N*-ethylmaleimide, but not towards iodoacetate and iodoacetamide. The SH_{II} group is unreactive except towards phenylmercuric ions. Both groups are reactive towards all reagents in the presence of 0.3% sodium dodecyl sulfate or 8 M urea.

These observations led to the preparation of two well-defined S-substituted lipase derivatives: mono 5-thio-2-nitrobenzoic acid (TNB) lipase in which the SH_I group was selectively blocked by a TNB radical and diphenyl mercuric (DPM) lipase in which the SH_I and SH_{II} groups were both blocked by a phenylmercuric radical. A "mixed" derivative containing a TNB radical on the SH_I group and a phenylmercuric radical on the SH_{II} group could also be prepared by incubation of mono TNB-lipase with phenylmercuric chloride. All these reactions were found to be fully reversed by incubation with an excess of thiol.

An intramolecular thiol–disulfide reaction was observed to occur when mono TNB-lipase was treated with 0.3% sodium dodecyl sulfate or 8 M urea or, although more slowly, when a solution of lipase in these reagents was oxidized by air. This reaction resulted in the formation of an additional disulfide bridge in the lipase molecule.

The kinetic parameters K_m and v_{max} of the hydrolysis of emulsified triglycerides by mono TNB-lipase, DPM-lipase and native lipase were determined and compared. Substitution of the SH_I group by a TNB radical induced a 10-fold increase in K_m whereas v_{max} was unaffected. Substitution of both the SH_I and SH_{II} groups by a phenylmercuric radical induced a 40% decrease in v_{max} while K_m was unchanged. Lipase regenerated by thiols from the derivatives had exactly the same parameters as the native enzyme.

A possible interpretation of these data is that the SH_I group is at or near the site responsible for the attachment of lipase at hydrophobic interfaces whereas the SH_{II} group is near the catalytic site. The properties of both groups suggest that they are situated in an hydrophobic region of the molecule.

Abbreviations: TNB, 5-thio-2-nitrobenzoic acid radical attached to a sulfur atom by a disulfide bridge; DTNB, 5,5'-dithiobis(2-nitrobenzoic) acid; DPM, diphenylmercuric radical.

INTRODUCTION

Two molecular forms of lipase (L_A and L_B) have recently been obtained in a pure, lipid-free form from defatted porcine pancreas powder by a succession of chromatographic separations on DEAE-cellulose, Sephadex G-100 and CM-cellulose[1]. Both forms appear to have the same activity towards emulsions of triglycerides, a similar molecular weight (about 50 000) and also a similar amino acid composition[1]. Both contain 6 disulfide bridges and 2 free SH groups per mole[2].

The purpose of this paper is to show that one of these groups (the SH_I group) is reactive in the native enzyme towards most sulfhydryl reagents except iodoacetate and iodoacetamide, whereas the second (the SH_{II} group) is generally unreactive unless the enzyme is denatured by sodium dodecyl sulfate or urea. However, phenylmercuric ions are able to combine with the SH_{II} group in the native enzyme.

The preceding observations led to the preparation of two well-defined mono and di S-substituted derivatives of native lipase. The kinetic parameters of these derivatives were determined in order to evaluate the respective role of the SH_I and SH_{II} groups in the activity of the enzyme. Some of the results obtained have already been published in a preliminary note[2].

MATERIAL AND METHODS

Preparation of lipase and activity assays

Lipase was purified from defatted porcine pancreas powder as previously described[1]. Each preparation was made with 80 g of powder. Lipase L_B, which separated from the enzyme L_A upon chromatography on CM-cellulose and represented the most abundant form (about 80% of the total), was employed for most of the assays. The salt-free, lyophilized preparations were kept at $-20°$ for several weeks without any detectable inactivation.

Two different techniques for the determination of lipase activity were used according to the purpose of the experiment. For routine assays, activity was measured titrimetrically at pH 9.0 with the aid of a recording Radiometer pH-stat on an olive oil emulsion stabilized by arabic gum in the presence of an optimal concentration of bile salts[3]. When higher accuracy and reproducibility were required, especially for kinetic assays, the titration was performed at pH 8.0 with purified tributyrin as the substrate[4]. Zero-order and reproducible kinetics are known to be obtained with this substrate in the absence of emulsifiers and bile salts, provided that the titration vessel and the agitation of the tributyrin–buffer mixture are carefully standardized.

Estimation of protein

Proteins were estimated at 280 nm with a Zeiss spectrophotometer Model PMQ II. A determination of the extinction coefficient ($E^{1\%}_{1\,cm}$) of pure lipase L_A or L_B at this wavelength gave a value of 13.3. The protein concentration of the solutions was checked carefully by amino acid analysis. The extinction coefficient at 280 nm of the mono 5-thio-2-nitrobenzoic acid (TNB) lipase (see below) was slightly higher ($E^{1\%}_{1\,cm} = 14.2$). A molecular weight value of 50 000 was used throughout for calculation of the molarity of lipase solutions.

Titration of SH groups by 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) (refs. 5 and 6)

A solution (1 ml) containing about 1 mg of lipase in 0.4 M NaCl was placed in a 1-cm thermostated cuvette of a Zeiss Spectrophotometer Model PMQ II or a Gilford multiple absorbance recorder Model 2000. This solution was mixed at 25° with 0.1 ml of a 0.5 M Tris–HCl buffer (pH 8.0) and an approx. 20-fold molar excess of DTNB dissolved in 0.25 ml of a 50 mM Tris–HCl buffer (pH 8.0). When the titration was performed in the presence of sodium dodecyl sulfate, a 3% solution of the detergent in the 0.5 M buffer was prepared and 0.1 ml was added to 1 ml of the enzyme (about 1 mg) in 0.4 M NaCl. For experiments carried out in concentrated urea all the solutions (0.4 M NaCl, 0.5 M buffer and DTNB) were made 8 M in urea by addition of the suitable amount of the crystalline reagent. The absorbance of the mixtures against a blank without lipase was measured or recorded at 412 nm. The number of reacting SH groups was calculated from the maximal absorbance, using a molar extinction coefficient value of 13 600 for the liberated thiolate anion. This value, indicated by ELLMAN[5,6], was verified in the presence or absence of sodium dodecyl sulfate or urea with known solutions of pure cysteine or reduced glutathione. The rate constant of the second-order reaction was derived from the slope of the straight-line corresponding to Eqn. 1:

$$\text{Log}\frac{a_0 - x}{b_0 - x} = f(t) \qquad (1)$$

where a_0 and b_0 are, respectively, the initial concentration of the reagent and of the SH groups. x is the concentration of the thiolate ion at time t.

Titration of SH groups by p-chloromercuribenzoate or N-ethyl maleimide

These assays were performed according to methods which have already been described[7,8].

Alkylation by iodoacetate or iodoacetamide

Lipase (48 nmoles or 2.4 mg) in 4 ml of a 55 mM Tris–HCl buffer (pH 8.0) containing 0.36 MNaCl was incubated for 2 h in the dark at room temperature under nitrogen with a 130–150 molar excess of iodoacetate or iodoacetamide (final concentration of the reagent, about 1.5 mM). The resulting mixtures were desalted by filtration through a 1.5 cm × 20 cm Sephadex G-25 column and lyophilized. A portion corresponding to 20 nmoles or 1 mg of enzyme was treated with DTNB in order to evaluate the number of remaining free SH groups. The other portion was hydrolyed under vacuum for 24 h in triple-distilled HCl and the amount of *S*-carboxymethylcysteine was evaluated with the aid of a Spinco–Beckman amino acid analyzer. The most stable amino acids, aspartic acid, glutamic acid, proline, glycine and alanine, were used as reference. In some assays, 0.3% sodium dodecyl sulfate was added during incubation of the protein with the reagent and during the subsequent gel filtration.

Reaction of native lipase with phenylmercuric ions

Phenylmercuric chloride or acetate (Aldrich) was recrystallized twice from absolute ethanol. A 6.8-mM solution (1 ml) of the reagent in pure methoxy-2-ethanol (methylcellosolve) was added to 10 ml of a 6.8 mg/ml solution of lipase in a 2.5 mM

Tris–HCl buffer (pH 9.0). After 30 min at room temperature, the mixture was desalted by passage through a 94 cm × 2.5 cm Sephadex G-25 column equilibrated with a 5 mM Tris–HCl buffer (pH 8.0) and lyophilized. An aliquot of the compound was incubated further with DTNB in order to evaluate the number of remaining free SH groups.

Synthesis of tris (α-bromopropionyl)- and tris (α-bromobutyryl)glycerol

Anhydrous glycerol was heated at 150° for 14 h under a stream of dry nitrogen with a 10% molar excess of the α-bromo acids (Fluka) in the presence of 1% *p*-toluene sulfonic acid. The mixture was washed twice with 10% bicarbonate and 3 times with distilled water. The resulting neutral product was dried over anhydrous Na_2SO_4, dissolved in a diethyl oxide–hexane mixture (60:40, v/v) and purified by chromatography on silicic acid. The overall yield was 30%. The identity of the compounds and their purity were checked by infrared spectrophotometry and thin-layer chromatography on silicic acid (20 cm × 30 cm plates; solvent system: hexane–diethyl oxide–formic acid (75:75:0.1, v/v/v).

RESULTS

Reactivity of the SH groups in native and denatured lipase

When native lipase was incubated at pH 8.0 or 9.0 with DTNB under the conditions described in the preceding section, a second-order thiol–disulfide exchange reaction was observed to occur. This reaction resulted, as indicated by Fig. 1, in the liberation of exactly 1 mole of 5-thio-2-nitrobenzoic acid per mole of enzyme. The apparent rate constants were 295 $moles^{-1} \cdot min^{-1}$ at pH 8.0 and 756 $moles^{-1} \cdot min^{-1}$ at pH 9.0. These values are much lower than those obtained under the same conditions with cysteine or reduced glutathione. Indeed, the reaction of DTNB with these latter compounds is so fast that the constants cannot be measured by ordinary techniques.

The liberation of 1 mole of 5-thio-2-nitrobenzoic acid per mole of native lipase suggested that a S-mono TNB derivative was formed according to Eqn. 2:

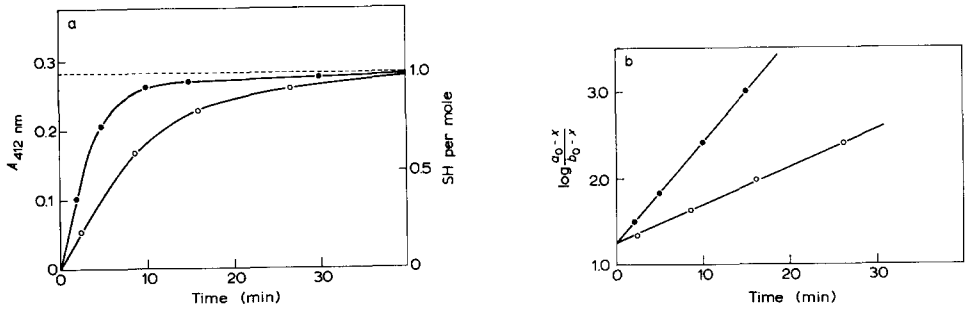
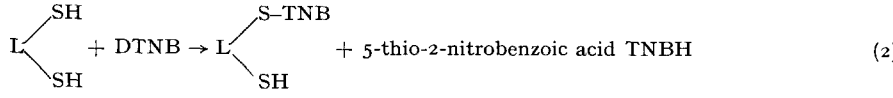


Fig. 1. Reaction of the SH_1 group in native lipase with DTNB. A 20-μM lipase solution (1.35 mg in 1.35 ml) was incubated at pH 8.0 (○—○) or pH 9.0 (●—●) and 25° with a 18-fold molar excess of DTNB. The liberated 5-thio-2-nitrobenzoic acid was measured by spectrophotometry at 412 nm. Further experimental details are given in MATERIAL AND METHODS. a. Variation of the absorbance as a function of time. b. Determination of the rate constant of the second-order reaction.



(2)

By contrast, two SH groups, designated hereafter SH_I and SH_{II}, were titrated by DTNB when the reaction was performed in 0.3% sodium dodecyl sulfate or 8 M urea (Fig. 2). Fig. 2 also shows that the non-ionic detergents Triton X-100 and Tween-80, when employed at the same concentration as sodium dodecyl sulfate, were unable to unmask the SH_{II} group. The effect of 0.3% sodium deoxycholate or sodium dodecanoate was very weak.

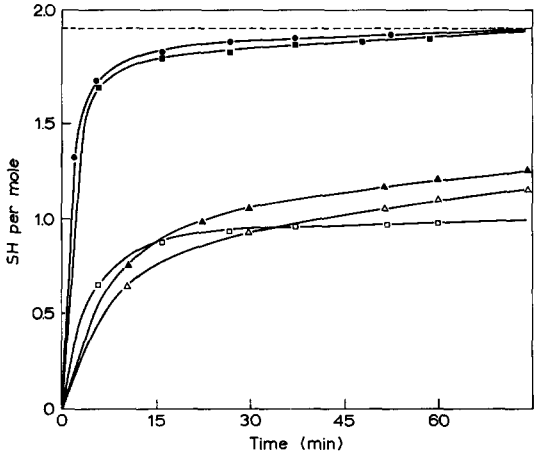


Fig. 2. Titration of SH groups by DTNB in the presence of various compounds. The titration was made in the presence of 0.3% sodium dodecyl sulfate (●—●), 8 M urea (■—■), 0.3% Triton X-100, 0.3% Tween-80 or 6 M urea (□—□), 0.3% sodium deoxycholate (▲—▲) or 0.3% sodium dodecanoate (△—△).

The course of the unmasking of the SH_{II} group by sodium dodecyl sulfate as a function of the detergent concentration is indicated in Fig. 3. This unmasking begins for concentrations below the critical micelle concentration of the detergent and it is complete a little before 0.3%. Addition of sodium dodecyl sulfate also induced a progressive inactivation of lipase.

The presence of one reactive SH group in porcine lipase was confirmed by titration of the native enzyme with *p*-chloromercuribenzoate and *N*-ethylmaleimide. The values found were, respectively, 0.98 and 0.80 SH group per mole of enzyme.

Moreover, alkylation of the SH groups by iodoacetate or iodoacetamide was attempted in the presence or absence of 0.3% sodium dodecyl sulfate. The number of S-alkylated cysteine residues was ascertained, either by direct estimation of the *S*-carboxymethylcysteine formed during alkylation, or by titration of the remaining SH groups with the aid of DTNB in the presence of 0.3% sodium dodecyl sulfate. The results reported in Table I show that the SH_I and SH_{II} groups are fully resistant in native lipase to alkylation at pH 8.0 by a large excess of the two reagents. By contrast, about 1.6 SH are readily alkylated after treatment of the enzyme by 0.3% sodium dodecyl sulfate.

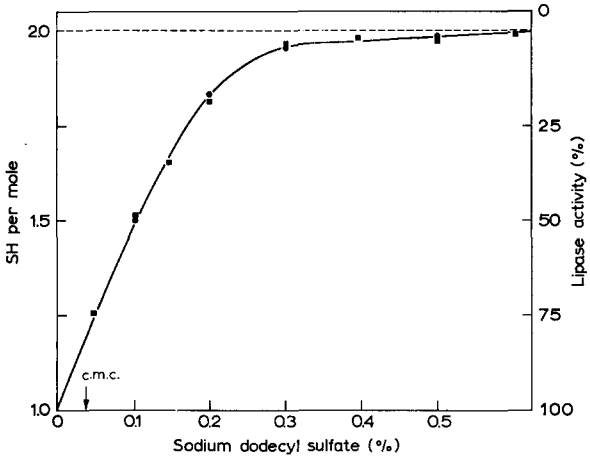


Fig. 3. Dependence of the unmasking of the SH_{II} group on sodium dodecyl sulfate concentration. Sulfhydryl groups in a 1 mg/ml lipase solution were titrated by DTNB after a 15-min incubation with varying concentrations of sodium dodecyl sulfate. Lipase activity was also measured in the incubated mixtures. ●—●, sulfhydryl groups; ■—■, lipase activity; c.m.c., critical micelle concentration.

Alkylation of the essential histidine in bovine ribonuclease by α-halogeno compounds has been found to require the presence in the alkylating agent of a free carboxylate reminiscent of the group carried by the nucleic acid substrates of the enzyme[9]. In an attempt to increase in a similar way the affinity of the agent for lipase, alkylation assays were performed with α-bromopropionic acid, α-bromobutyric acid and the glycerides of these acids (tris-(α-bromopropionyl)glycerol and tris-(α-bromobutyryl)glycerol). Negative results were obtained in all cases. Emulsions of the bromoglycerides in 0.1 M NaCl were observed to be hydrolyzed at pH 8.0 by lipase at a rate

TABLE I

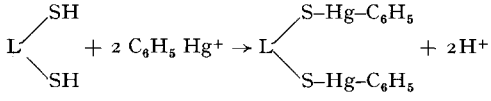
ALKYLATION OF SH GROUPS OF LIPASE WITH IODOACETATE OR IODOACETAMIDE

Alkylating agent	*Molar excess of the reagent*	*Lipase*	*Number of alkylated SH groups in 1 mole lipase*	
			Titration by DTNB	*Determination of carboxymethyl-cysteine*
Iodoacetate	150	Native	0.0	0.0
Iodoacetamide	130	Native	0.0	0.0
Iodoacetamide (in 10 mM EDTA)	130	Native	0.2	0.0
Iodoacetate	130	Denatured (0.3% sodium dodecyl sulfate)	1.5	1.25–1.38*
Iodoacetate	130	Pepsic hydrolysate	1.7	1.25–1.30*

* Not corrected for losses. These losses were estimated to be about 20% by assays performed under identical conditions with reduced glutathione.

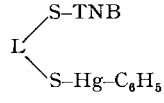
not exceeding 1% of that measured on triolein. In agreement with previous observations[10,11], the bulky bromine atom adjacent to the ester bond appears to exert an unfavorable effect on lipase action.

On the other hand, *p*-chloromercuribenzoate, which was reported earlier to have no action on the SH_{II} group in native lipase, can be advantageously replaced by phenylmercuric ions. Native lipase dissolved in a 2.5 mM Tris–HCl buffer (pH 9.0) was incubated at room temperature for 30 min with a 5 molar excess of phenylmercuric chloride or acetate in methylcellosolve. At the end of the treatment, the small molecules were removed from the mixture by passage through Sephadex G-25. No free SH groups could be detected by titration with DTNB in the presence of 0.3% sodium dodecyl sulfate. The conclusion was that the SH_I and the SH_{II} groups in native lipase had reacted, inducing the formation of a diphenylmercuric derivative (Eqn. 3). This new derivative, designated diphenylmercuric (DPM) lipase, will be more precisely identified later.



(3)

It is noteworthy in this connexion that incubation of mono TNB-lipase with phenylmercuric chloride under the same conditions gave rise to the "mixed" disubstituted derivative:



in which the SH_I and SH_{II} groups were blocked, respectively, by one TNB and one phenylmercuric radical.

Preparation and properties of mono TNB-lipase

Pure mono TNB-lipase was prepared by incubation of 100 mg of a mixture of lipases $L_A + L_B$ with DTNB. The liberation of the expected stoichiometric quantity of thionitrobenzoic acid was verified by spectrophotometry and the solution was freed from low molecular weight contaminants by passage through a Sephadex G-100 column equilibrated with 0.4 M NaCl. The protein fractions were pooled, dialyzed overnight against distilled water and lyophilized. The powder was kept at −20°.

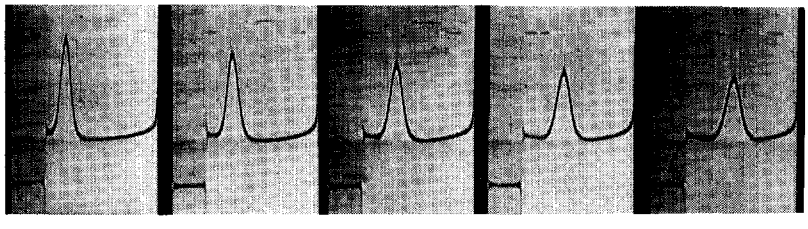


Fig. 4. Sedimentation diagram of mono TNB-lipase L_B. A 8 mg/ml solution of the derivative in a 0.5 M Tris–HCl buffer (pH 8.0) was analyzed at 59 570 rev./min and 25° in a Spinco–Beckman ultracentrifuge Model E. Photographs were taken 16, 24, 32 and 48 min after the rotor had reached its maximal speed. Under these conditions, the sedimentation constant of the derivative was found to be 4.0 S.

This powder was dissolved in 50 ml of a 50 mM sodium acetate–acetic acid buffer (pH 5.0) and the solution was submitted to chromatography in a 45 cm × 2.5 cm CM-cellulose column equilibrated with the same buffer. After a preliminary washing with 75 ml of the buffer, the column was eluted by the same linear increase of pH as for the separation of lipases L_A and L_B (ref. 1). Two peaks, corresponding respectively to the mono TNB derivatives of lipases L_A and L_B were separated. Both were found homogeneous by disc electrophoresis (7.5% gel, pH 8.5) and by ultracentrifugation. The sedimentation diagram of mono TNB-lipase L_B is reproduced in Fig. 4.

Moreover, Sephadex filtration assays demonstrated that the preparations contained no detectable traces of dimers or higher polymers. The amino acid composition of the derivatives was determined by the usual technique and found to be identical to that already reported[1] for the corresponding enzymes.

The spectrum of the TNB radical in DTNB with its characteristic maximum at 324 nm is presented in Fig. 5c. A similar absorbance peak with a maximum at approx. 325 nm is also visible in Fig. 5b related to mono TNB-lipase. Assuming that the TNB radical had the same molar extinction coefficient (9050) in DTNB and in mono TNB-lipase, this latter could be shown to contain 0.90–1.05 TNB radical per mole. All these data definitely proved that the isolated compound was actually a monosubstituted derivative in which a single TNB radical was attached by an S–S bridge to the SH_I group of the enzyme.

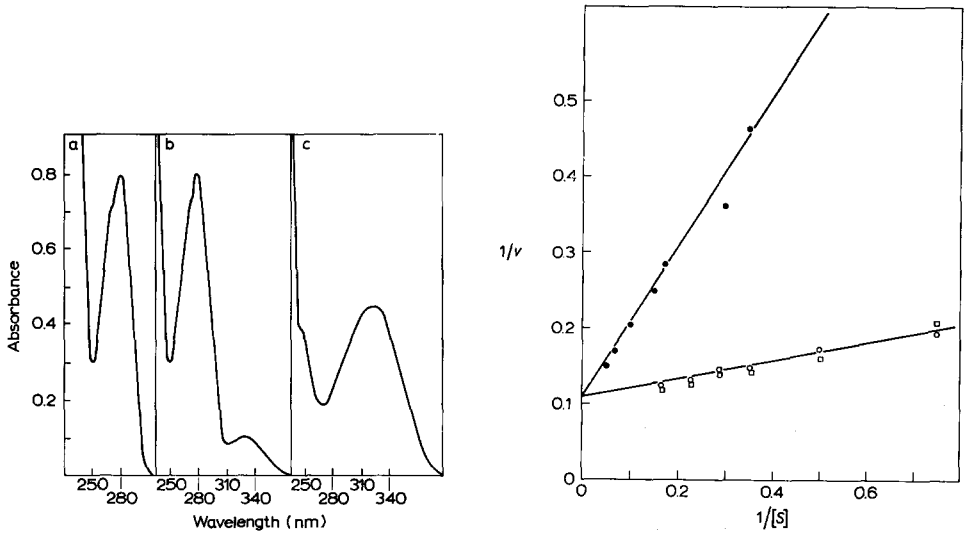
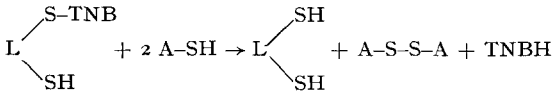


Fig. 5. Ultraviolet spectra of native and mono TNB-lipase L_B and DTNB. Ultraviolet spectra were obtained with a Cary Model 14 recording spectrophotometer. Solutions were made in 0.4 M NaCl. Temp., 25°. (a) Lipase L_B (0.6 mg/ml). (b) Mono TNB-lipase L_B. (c) DTNB, 9.8 mg/ml or 2.48 μM.

Fig. 6. Lineweaver–Burk plot of the hydrolysis of tributyrin emulsions by native, mono TNB-, and regenerated lipase. The experimental conditions of the assays using dispersed tributyrin as substrate are those described in ref. 4. v_{max} is expressed in μequiv of butyric acid liberated per min under the conditions of the test. The concentration of the emulsified substrate is not expressed in m^2 of interface per l of emulsion[12], but more simply in g of substrate per 100 ml emulsion. This expression is valid provided that the operations are carried out under carefully standardized conditions. ○—○, native lipase; ●—●, mono TNB-lipase; □—□, regenerated lipase.

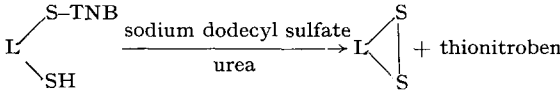
Moreover, the reaction of lipase with DTNB was observed to be easily reversed by addition of an excess of thiol (A–S$^-$) according to Eqn. 4:



(4)

Mono TNB-lipase (1 mg) in 1 ml of 0.4 M NaCl was placed in the cuvette of a Cary-14 spectrophotometer with 0.1 ml of a 0.01 M solution of cysteine (or reduced glutathione or mercaptoethanol) in a 0.5 M Tris–HCl buffer (pH 8.0). The absorbance increase at 412 nm indicated a stoichiometric liberation of 1 mole of thionitrobenzoic acid per mole of derivative.

A quite interesting intramolecular thiol–disulfide exchange reaction (Eqn. 5) was also found to occur when 0.3% sodium dodecyl sulfate or 8 M urea was added to a mono TNB-lipase solution in a 50 mM Tris–HCl buffer (pH 8.0)



(5)

The formation of a new derivative of lipase with an additional disulfide bridge  or "SS-lipase") was demonstrated by the following observations: (a) exactly 1 mole of thionitrobenzoic acid per mole of mono TNB-lipase was released after a 40–45 min incubation with one of the above-mentioned reagents; (b) after filtration of the resulting solutions through Sephadex G-25 equilibrated with the buffer 0.3% in sodium dodecyl sulfate, no free SH could be titrated by DTNB. No absorbance peak at 325 nm could also be observed; (c) the electrophoretic migration of the compound in the presence of 0.3% sodium dodecyl sulfate[9] was consistent with a molecular weight of about 50 000. The SH_{II} group was also observed to disappear completely within approx. 3 h when air was bubbled through a solution of lipase in 0.3% sodium dodecyl sulfate at pH 8.0. It was, therefore, likely that the additional S–S bridge could be formed by direct oxidation after unmasking of the SH_{II} group.

Finally, the kinetic parameters K_m and v_{max} of native lipase were compared to those of mono TNB-lipase and of the enzyme regenerated from this derivative. Fig. 6 shows that the K_m of mono TNB-lipase is 10 times higher than the K_m of the native enzyme whereas v_{max} is the same for both forms. By contrast, the two parameters have exactly the same value for the regenerated and for the native enzyme. Hence, the specific reaction of the SH_I group with DTNB can be considered as fully reversible.

Preparation and some properties of DPM-lipase

It was reported earlier that *p*-chloromercuribenzoate reacted only with the SH_I group of native lipase whereas the more hydrophobic phenylmercuric ions were able to react with the two groups.

The derivative resulting from this latter reaction was prepared by incubation at room temperature for 30 min of 10 ml of a 6.8 mg/ml aqueous solution of lipase L_B (1.36 μmole), 1 ml of 25 mM Tris–HCl buffer (pH 9.0) and 1 ml of a 6.8-mM solution

of phenylmercuric chloride or acetate (6.8 μmoles) in freshly-distilled methylcellosolve. The adopted 5-fold molar excess of reagent over the enzyme will be justified later. The incubated mixture was freed from low molecular weight contaminants by filtration through a 94 cm × 2.5 cm Sephadex G-25 column equilibrated with a 5 mM Tris buffer (pH 8.0). The protein fractions were pooled, exhaustively dialyzed against water and lyophilized. No traces of free SH groups could be detected in this preparation by titration with DTNB in the absence or in the presence of sodium dodecyl sulfate. The derivative migrated exactly as native lipase during Sephadex G-100 filtration and during disc electrophoresis. It was definitely identified as the S-DPM derivative of lipase (DPM-lipase) by direct evaluation of its mercury content by atomic adsorption with the aid of a Eel apparatus equipped with a mercury lamp emitting at 253.7 nm. A value of 2.1 mercury atom per mole of derivative was found. Moreover, a lipase indistinguishable from the native form could be obtained by treatment of DPM-lipase by 1 mM mercaptoethanol and filtration of the resulting product through Sephadex G-25. Hence, the phenylmercuric ions are able, like DTNB, to realize a highly specific and fully reversible substitution in native lipase.

Since phenylmercuric ions react with the SH_{II} group in native lipase, they provide an interesting opportunity for evaluating the possible role of this group in the activity of the enzyme. Fig. 7 indicates that incubation of lipase with the ions induces a marked inhibition. With a molar excess of reagent as low as 5-fold, a sharp activity decrease of about 40% during the first min followed by a plateau is observed. When a larger excess (25- or 50-fold) is employed, further inactivation occurs at a much slower rate after the same initial drop.

Since the SH groups are known to interact most readily with mercuric compounds, a plausible assumption is that a fast reaction occurs between the SH_I and SH_{II} groups of lipase and the phenylmercuric ions. This reaction would partially inactivate the enzyme and it would be followed, when the reagent is in large excess, by other non-specific processes. This assumption was confirmed by the finding that no SH groups could already be titrated in 40% inactivated lipase at the end of the

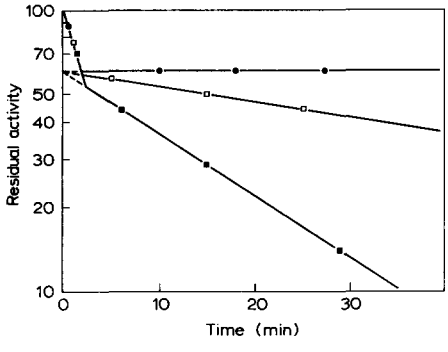


Fig. 7. Inhibition of lipase L_B by varying concentrations of phenylmercuric chloride. Lipase was treated by a solution of phenylmercuric chloride in methylcellosolve (see text). Methylcellosolve alone induced no inactivation. Lipase activity was measured at pH 9.0 against a triolein emulsion. The molar excess of reagent over lipase was 5- (●—●), 25- (□—□) or 50- fold (■—■).

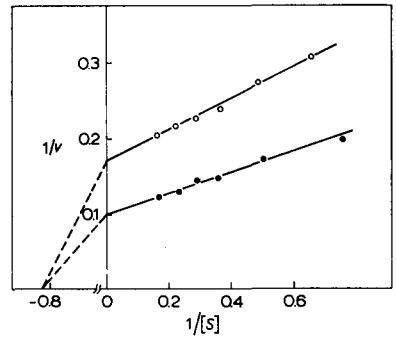


Fig. 8. Lineweaver–Burk plot of the hydrolysis of tributyrin by native and DPM-lipase. The general conditions are the same as in the experiments of Fig. 5. ○—○, DPM-lipase; ●—●, native lipase.

initial period. Consequently, a convenient technique for the preparation of DPM-lipase is to use a molar excess of phenylmercuric ions not exceeding 5-fold.

A comparison between the kinetic parameters of native lipase and DPM-lipase is presented in Fig. 8. K_m is seen to be unaffected by the modification of the SH_I and SH_{II} groups. Conversely, v_{max} is 40% smaller. Removal of the organomercuric radicals by mercaptoethanol regenerated a lipase possessing a v_{max} identical to that of the native enzyme. A last observation was that the v_{max} of the reaction catalyzed by the "mixed" derivative described earlier was also 60% active. The K_m of this reaction was not determined.

DISCUSSION

Several years ago, crude preparations of pancreatic lipase were claimed to be inhibited by classical sulfhydryl reagents such as α-bromoacetate, α-iodoacetate and *p*-chloromercuribenzoate[13]. This led to the suggestion that the enzyme contained essential SH groups[14,15]. However, more recently the partially purified enzyme was shown by WILLS[16] to be completely resistant to 1 mM iodobenzoate or iodoacetate. Lipase was also found to be 70% inhibited by 1 mM *p*-chloromercuribenzoate and to be extremely sensitive to low concentrations of iodine and Cu^{2+}. However, the author pointed out that inhibition by iodine was not reversed by thiols and that Cu^{2+} could bind at sites other than sulfhydryls in the enzyme molecule, for instance at imidazole rings. The conclusion was that lipase was likely to possess SH groups and that these groups were situated adjacent to, but not in, the active site of the enzyme.

Two SH groups with widely different reactivities have now been shown to exist in pure porcine pancreatic lipase and two chemically well-defined S-substituted derivatives of the native enzyme have been prepared.

The fact that DPM-lipase is still 40% as active as the native enzyme under the experimental conditions used in our test, definitely rules out the possibility for lipase to be a "sulfhydryl enzyme". Recent inhibition assays performed in this laboratory with the aid of diethyl *p*-nitrophenyl phosphate (ref. 17 and unpublished experiments of Dr. M. F. MAYLIÉ) and by photo-oxidation[4] are consistent with the view that, as for a number of esterases, the catalytic site of lipase is of the serine–histidine type.

However, it is noteworthy that the substitution of the SH groups with various radicals affects the kinetic parameters of the lipase-catalyzed hydrolysis of emulsified triglycerides and that this effect is different according to the nature of the radical and/or the nature of the group concerned. Substitution of the SH_I group by a TNB radical induces a substantial increase of K_m whereas substitution of both the SH_I and SH_{II} groups by a phenylmercuric radical is associated with a moderate decrease of v_{max}. Although the K_m is not an association constant in the case of lipase[4], a possible explanation of the data would be that the SH_I group is at or near the binding site and that the attachment of the enzyme at hydrophobic interfaces is quite noticeably affected by the presence of a carboxylate in the TNB radical bound to the SH_I group. The eventuality for the SH_I group to play a role in the formation of the lipase–substrate complex is corroborated by the observation that colipase is more easily separated from mono TNB-lipase than from lipase itself[18]. In the same connexion, it may be postulated that the SH_{II} group is situated near the catalytic site of the

enzyme, so that any substitution of an hydrogen atom in the group by a bulky radical lowers the efficiency of this site. Quite obviously, this explanation is only tentative. But, the finding that none of the SH groups are reactive towards α-halogeno acids in native lipase and that the SH_{II} group, which does not react with DTNB or *p*-chloromercuribenzoate, is accessible only to phenylmercuric ions suggests that the SH groups are normally situated in an hydrophobic region of the enzyme molecule.

The structure in native lipase responsible for the large difference in reactivity existing between the SH_I and the SH_{II} groups as well as the cause of the unmasking of the SH_{II} group by sodium dodecyl sulfate or urea remains to be elucidated. This unmasking does not take place unless the urea concentration exceeds 6 M and it is complete in 8 M urea. By contrast, Fig. 3 shows that the unmasking of the SH_{II} group already starts for quite low concentrations of sodium dodecyl sulfate and that it steadily increases until 0.3%. The good correlation seen in Fig. 3 between inactivation of the enzyme and unmasking of the SH_{II} group suggests that both processes have a common cause which can be assumed to be the irreversible denaturation of the protein. In this respect, it will be interesting to study by suitable techniques the effect of sodium dodecyl sulfate or urea on the conformation of lipase in solution. It should also be pointed out that the curves in Fig. 3 do not indicate that sodium dodecyl sulfate micelles are more efficient for the unmasking of the SH_{II} group than monomers. This is in accordance with the results obtained by REYNOLDS AND TANFORD[19,20] on other proteins with the aid of different techniques.

A last point of interest is that, upon treatment of mono TNB-lipase by sodium dodecyl sulfate and urea or upon air oxidation of a lipase solution in one of these reagents, a thiol–disulfide exchange reaction takes place leading to the formation of a new S–S bridge in the molecule. This strictly intramolecular reaction requires that the SH groups are correctly positioned with respect to one another. Then, two hypothesis may be formulated: Either the positions of the groups are already correct in native lipase and the sole effect of the denaturing agents would be to "activate" the SH_{II} group by some unknown mechanism. Or, the groups are brought to the correct positions as a result of the conformational changes and/or the increased flexibility of the protein backbone associated with denaturation.

ACKNOWLEDGEMENTS

Our thanks are due to Miss F. Escudero for her expert technical assistance and to Miss J. Philip for performing the analytical electrophoresis in the presence of sodium dodecyl sulfate. Infrared spectrophotometric assays were kindly performed by Dr. R. Julien at the Technical Center of Infrared Spectroscopy of the University (Professor J. Chouteau). The atomic adsorption assays were kindly performed by Dr. M. F. Maylié in the Centre de Biochimie et de Biologie Moléculaire du C.N.R.S. (Marseille).

REFERENCES

1 R. VERGER, G. DE HAAS, L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 188 (1969) 272.
2 R. VERGER, L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 207 (1970) 377.
3 P. DESNUELLE, M. J. CONSTANTIN AND J. BALDY, *Bull. Soc. Chim. Biol.*, 37 (1955) 285.
4 M. SÉMÉRIVA, C. DUFOUR AND P. DESNUELLE, *Biochemistry*, 10 (1971) 2143.

5 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 74 (1958) 443.
6 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
7 P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 4331.
8 J. F. RIORDAN AND B. L. VALLEE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods of Enzymology*, Academic Press, New York, 1967, p. 545. Vol. XI,
9 G. R. STARK, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 236 (1961) 436.
10 H. BROCKERHOFF, *Biochim. Biophys. Acta*, 212 (1970) 92.
11 S. BERGSTROM, B. BORGSTROM, N. TRYDING AND G. WESTOO, *Biochem. J.*, 58 (1954) 604.
12 G. BENZONANA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 105 (1965) 121.
13 S. S. WEINSTEIN AND A. M. WYNNE, *J. Biol. Chem.*, 112 (1936) 641.
14 T. P. SINGER AND E. S. G. BARRON, *J. Biol. Chem.*, 157 (1945) 241.
15 J. E. SCOTT, *Nature*, 172 (1953) 777.
16 E. D. WILLS, *Biochim. Biophys. Acta*, 40 (1960) 481.
17 M. F. MAYLIÉ, M. CHARLES, L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 178 (1969) 196.
18 M. F. MAYLIÉ, M. CHARLES, C. GACHE AND P. DESNUELLE, *Biochim. Biophys. Acta*, 229 (1971) 286.
19 J. A. REYNOLDS AND C. TANFORD, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 1002.
20 J. A. REYNOLDS AND C. TANFORD, *J. Biol. Chem.*, 245 (1970) 5161.