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Importance of sulfhydryl group for rabbit gastric lipase activity

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We have shown recently that rabbit gastric lipase (RGL) purified from gastric tissue presents catalytic properties comparable with those of human gastric lipase (HGL). We report here that only one sulfhydryl group was modified per molecule of native RGL after incubation at pH 8.0 with 5,5'-dithiobis(2-nitrobenzoic acid) (NbS2) for 4 h or 4,4'-dithiopyridine (4-PDS) for 60 min. With both reagents, a direct correlation was found between the modification of one sulfhydryl group per enzyme molecule and loss of RGL activity. Incubation of RGL with the new hydrophobic sulfhydryl reagent, dodecyldithio-5-(2-nitrobenzoic acid) (C12-NbS), at 30-fold molar excess, at pH 3.0, 5.0 and 8.0, induced immediate and complete inactivation of RGL. Unlike NbS2 and 4-PDS, C12-NbS almost instantaneously stopped the course of tributyrin hydrolysis by RGL, in contrast to porcine pancreatic lipase (PPL). RGL can be included with HGL in the group of sulfhydryl enzymes.

Lipase; Sulfhydryl enzyme; Chemical modification; Binding; Catalysis

1. INTRODUCTION

In humans, several conditions including cystic fibrosis, pancreatitis, premature birth and alcoholism are associated with pancreatic lipase insufficiency. In order to palliate this insufficiency, enzyme-replacement therapy with acid stable lipases has been suggested as a method of treatment. We have recently screened the presence of preduodenal lipases in 11 mammals [1]. The properties of all preduodenal lipase activities detected differ markedly from those of pancreatic origin.

Rabbit stomach is a potential abundant source of acid-stable lipase and this enzyme could be an interesting model for both fundamental studies concerning the physiological importance of preduodenal lipases and possible therapeutic applications.

Recently, we have purified a lipase from rabbit gastric tissue (RGL) [2]. The presence of amphiphiles such as BSA or bile salts prevents its irreversible denaturation [2]. The N-terminal sequence of RGL reveals marked homology with that of HGL [2].

The aim of the present study was to determine the number of free sulfhydryl groups in RGL, as well as their reactivity and contribution to the expression of lipase activity. Results are compared with data previously obtained with HGL and PPL.

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Abbreviations: RGL, rabbit gastric lipase; HGL, human gastric lipase; PPL, porcine pancreatic lipase; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; NbS, 5-thio(2-nitrobenzoic acid); NbS2: 5,5'-dithiobis(2-nitrobenzoic acid); C12-NbS, dodecyl dithio-5-(2-nitrobenzoic acid); 4-TP, 4-thiopyridone; 4-PDS, 4,4'-dithiopyridine; PMC, phenyl mercuric chloride; tributyrin, tributyrylglycerol

2. MATERIALS AND METHODS

2.1. *Lipids*

Tributyrin (puriss) was obtained from Fluka (Buchs, Switzerland) and taurodeoxycholate (TDC) from Sigma (St. Louis, MO).

2.2. *Lipase and proteins*

RGL was purified to electrophoretic purity from gastric tissue as in [2]; BSA was from Sigma.

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2.3. *Protein concentration*

BSA concentration was determined spectrophotometrically at 280 nm using an absorption coefficient of $E = 6.7$. RGL concentration was determined according to Lowry et al. [3].

2.4. *RGL activity measurements*

RGL activity was measured titrimetrically at pH 6.0 and 37°C with a pH-stat apparatus (TTT 80, Radiometer, Copenhagen) using a tributyrin emulsion as substrate: 0.5 ml tributyrin added to 14.5 ml of 150 mM NaCl, 2 mM TDC and 2 μM BSA [4].

2.5. *Sulfhydryl reagents*

NEM, PMC, NbS2 and 4-PDS were purchased from Aldrich (Strasbourg), 1-dodecanethiol being from Janssen (Pantin, France).

2.6. *Chemical synthesis of C12-NbS*

Dodecanethiol (15 mmol) and NbS2 (20 mmol) were dissolved in 3 l methanol/chloroform (2:1, v/v). This mixture was stirred continuously for 24 h at 25°C in the dark. After adding 1 l chloroform and 1 l water, the organic lower phase was recovered and the solvent evaporated. The remaining products were dissolved in 5 ml hexane. C12-NbS was purified by chromatography on a silicic acid column (5 cm diameter, 7 cm height). The products were eluted by successive volumes (100 ml hexane/diethyl ether/acetic acid ranging from 90:10:1 to 50:50:1, by vol.). C12-NbS was pure as determined by thin-layer chromatography using hexane/diethyl ether/acetic acid (60:40:1, by vol.) as solvent.

2.7. *Reaction of RGL with NbS2 or 4-PDS*

NbS2 and 4-PDS are commonly used reagents for spectrophotometric determination of sulfhydryl groups in biological materials [5,6]. NbS2 is not useful under acidic conditions for 2 reasons: the NbS product is only chromophoric in the dianionic form (>98% above pH 7) and NbS2 has been shown not to react with cysteine at pH 3.3. 4-PDS, on the other hand, has been shown to give quantitative results between pH 3.4 and 8.1. A 1 ml spectrophotometry cuvette was filled with 1 ml of 0.25 M Tris-HCl buffer (pH 8.0), containing RGL (0.5–1 mg) and a 40–60-molar excess of NbS2 or 4-PDS. The value of the absorbance of the mixtures as compared with a blank containing no RGL was recorded vs time. The number of sulfhydryl groups was calculated from the maximal absorbance, using a value for the molar extinction coefficient of 13 600 (at 412 nm) for NbS and 19 800 (at 324 nm) for thiopyridone. At the same time, residual HGl activity was measured on samples. In some experiments, SDS (3%, w/w, final concentration) was added.

2.8. *Kinetic parameters*

Rate constants were calculated from the rates of NbS or 4-TP release, and for inactivation. Even though we used an excess of sulfhydryl reagents, the pseudo-first-order treatment did not give a linear fit of the data. In both cases, second-order rate constants of the reactions were obtained from the slope of the straight line corresponding to the equation:

$$\log\left(\frac{a_0-x}{b_0-x}\right) = k(a_0-b_0)t + \log\frac{a_0}{b_0}$$

where a_0 is the initial concentration of reagent, b_0 represents the RGL concentration and x corresponds to the concentration of modified sulfhydryl group or of inhibited enzyme at time t. k represents the rate constant for either sulfhydryl group modification (k_{SH}), or RGL inhibition (k_i).

3. RESULTS

3.1. *NbS2 and 4-PDS reaction with sulfhydryl group of RGL*

When RGL was incubated, at pH 8.0, with NbS2 (fig.1A) or 4-PDS (fig.1B), release of NbS or 4-TP ions was observed.

During incubation with NbS2, 1 mol NbS ion per mol enzyme was detected after 4 h (fig.1A). With 4-PDS the reaction proceeded more rapidly and 1 mol 4-TP was released per mol RGL after 1 h of incubation. With both reagents, only 1 mol sulfhydryl group per mol RGL was immediately detected in the presence of 3% SDS (w/w, final concentration) (not shown). Fig.1A,B demonstrates for both reagents the parallel between the kinetics of RGL inhibition and the number of sulfhydryl groups modified per molecule of RGL.

The data given in fig.1A,B were plotted according to the equation:

$$\log\frac{a_0-x}{b_0-x} = f(t)$$

and used to determine the rate constants (fig.1C,D). The values of these rate constants are listed in table 1. The rate of reaction of 4-PDS with RGL can be seen to be about 8-times faster than

Table 1

Reaction constants

Lipase	Rate constants ($M^{-1}\cdot min^{-1}$)	Reagents	
		DTNB	4-PDS
RGL	k_{SH}	9.1 ±0.5	68.1 ± 7.3
	k_i	8.1 ±0.5	70.3 ± 3.6
HGL[a]	k_{SH}	2.85 ± 0.09	25.6 ± 0.9
	k_i	3.76 ± 0.16	23.2 ± 0.9
PPL[b]	k_{SH}	295	n.d.

[a] Result from [7]

[b] Result from [9]

kSH, rate constant for sulfhydryl group modification; k_i, rate constant for HGL inhibition; n.d., not determined

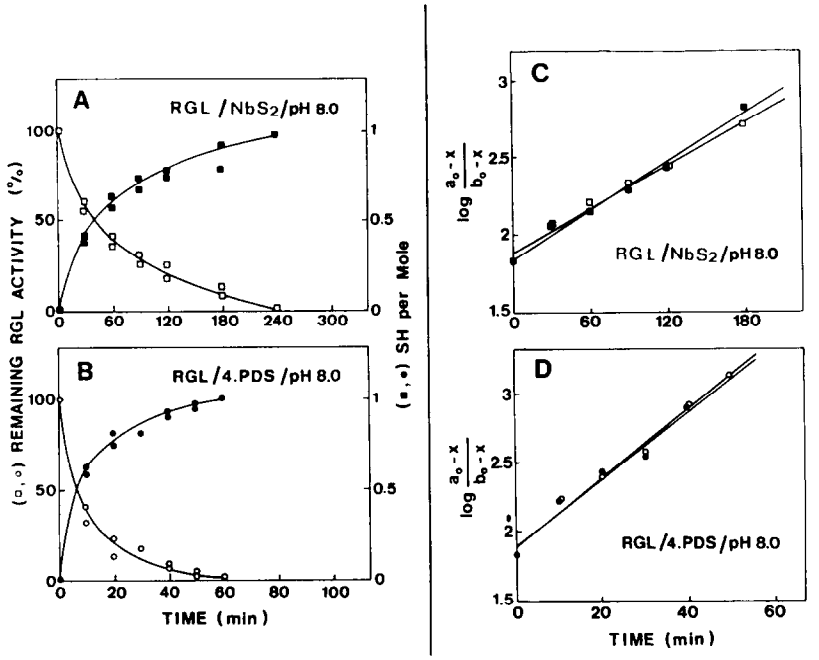

Fig.1. Variation in RGL activity and sulfhydryl group modification during incubation with sulfhydryl reagents: determination of the rate constants of sulfhydryl group modification. (A) RGL (20 nmol) was incubated at pH 8.0 and at 25°C with NbS2 (1333 nmol). The liberated NbS was measured spectrophometrically at 412 nm (E^{M}_{1cm} 13 600). The number of sulfhydryl groups modified per RGL molecule was calculated at different incubation times (■). In parallel, residual RGL activity was measured by tributyrin hydrolysis (□). (B) RGL (12 nmol) was incubated at pH 8.0 and 25°C with 4-PDS (833 nmol). The liberated 4-TP was measured spectrophotometrically at 324 nm (E^{M}_{1cm} 19 800). The number of sulfhydryl group modified per RGL molecule was calculated at various incubation times (●). Residual RGL activity was measured on tributyrin as substrate (○). The kinetic reactions shown in (1A,B) are plotted according the following equation:

$$\log \frac{a_0 - x}{b_0 - x} = k(a_0 - b_0)t + \log \frac{a_0}{b_0}$$

(C) Curves derived from (A); (■) reaction of DTNB with RGL; (□) inactivation of RGL during incubation with NbS2. (D) Curves derived from (B); (●) reaction of 4-PDS with RGL; (○) inactivation of RGL during incubation with 4-PDS. Lines are drawn after linear regression analysis. (correlation coefficient > 0.99).

that of NbS2. With both reagents, similar rate constants for enzyme inactivation and sulfhydryl modification were obtained. This finding is compatible with the existence of a direct correlation between the modification of one sulfhydryl group and the loss of HGL activity. As is evident from a comparison of the values of the rate constants (table 1), RGL reacts 3-times more rapidly than HGL with both reagents. Using NbS2 the reaction is 30-times slower with RGL than with PPL.

3.2. *Effect of pH on RGL inactivation by 4-PDS*

We measured the remaining RGL activity after incubation with 4-PDS at various pH values. Parallel control experiments without 4-PDS run for each assay showed no significant decrease in RGL activity, even after 72 h of incubation at all pH values tested.

Fig.2 shows that at pH values below 5.0, after 72 h, RGL activity was no longer significantly affected by the presence of 4-PDS. At higher pH values, RGL activity decreased rapidly. For instance, complete enzyme inactivation was observed after about 2 days at pH 7.0, whereas total inactivation was observed after only 1 h at pH 8.0.

The presence of one reactive sulfhydryl group

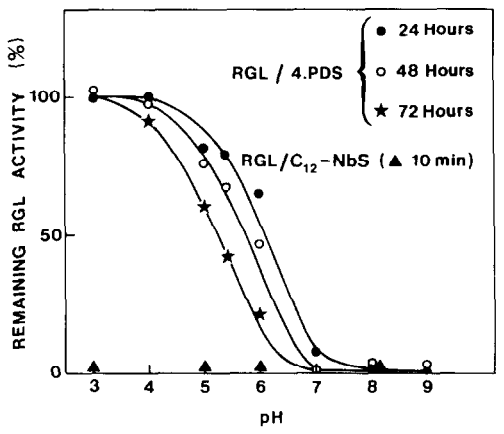

Fig.2. Variations in RGL activity during incubation with 4-PDS or C12-NbS at various pH values. RGL (9 nmol) was incubated at 25°C at various pH values with 4-PDS (660 nmol) for several periods of time: (●) 24 h, (○) 48 h and (*) 72 h. Residual RGL activity was measured on tributyrin as substrate. (▲) RGL activity remaining after 10 min of incubation with C12-NbS.

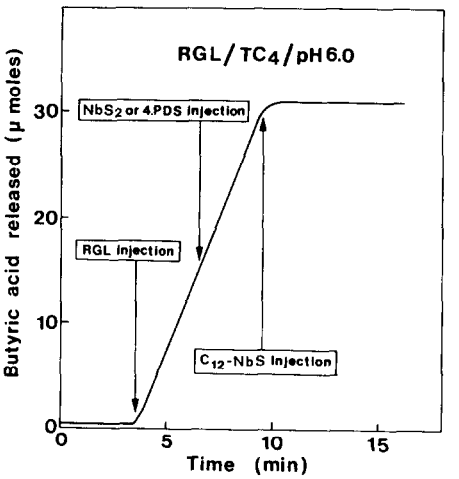

Fig.3. Effect of sulfhydryl reagents on RGL activity during the course of lipolysis. The three arrows indicate successive injections of RGL (13 nmol), NbS2 or 4-PDS (final concentration 0.12 mM) and then C12-NbS (final concentration 0.12 mM). The release of butyric acid was recorded continuously at 37°C and pH 6.0, using a pH-stat apparatus.

per RGL molecule was further confirmed by incubating the enzyme with NEM or PMC. A 500 molar excess of these sulfhydryl reagents was found to induce full inactivation of RGL after 4 h of incubation at pH 8.0 (not shown).

3.3. *Reaction of C12-NbS with the sulfhydryl group of RGL*

An incubation period (several hours) with NbS2 was needed to modify the essential sulfhydryl group of RGL (fig.1). In order to increase this reaction rate, we synthesized and tested a new sulfhydryl reagent, C12-NbS, which bears a hydrophobic moiety.

At pH 3.0, 5.0, 6.0 or 8.0, incubation of RGL with C12-NbS (molar ratio: 30) induces the rapid and complete inactivation of enzyme (fig.2). This inactivation parallels the release in the incubation medium of one NbS ion per RGL molecule (not shown). Further addition of SDS did not increase the number of NbS ions released per molecule of RGL (not shown).

3.4. *Effect of the addition of C12-NbS during the course of lipolysis*

In order to study the capacity of sulfhydryl reagents to inhibit RGL activity during the lipolysis of triacylglycerol, we successively injected into the emulsified tributyrin system at pH 6.0 a solution of NbS2, 4-PDS or C12-NbS (final concentration 0.12 mM for each reagent). As demonstrated in fig.3, the addition of NbS2 or 4-PDS after HGL injection had no effect on the course of tributyrin hydrolysis. In contrast, C12-NbS almost immediately stopped RGL activity. Addition of NbS2 or 4-PDS to the tributyrin emulsion before RGL injection did not influence the kinetics of hydrolysis, whereas C12-NbS prevents the action of lipase when added to the emulsified system prior to the enzyme (not shown).

4. DISCUSSION

To characterize further RGL and to compare its structural properties with HGL, we have investigated here the importance of the sulfhydryl groups. We have estimated that the accessible sulfhydryl group in RGL reacts 3-times faster than the corresponding residue in HGL [7] when NbS2 or 4-PDS is used as a sulfhydryl reagent under similar experimental conditions (see table 1). In contrast, using NbS2, RGL reacts 30-times slower than the corresponding residue in PPL (SH1) [8,9]. In view of the hydrophilic character of NbS2, it

can be tentatively concluded that the immediate spatial surroundings of the sulfhydryl group in HGL may be more hydrophobic than the SHI environment in PPL.

Modification of the SHI group in PPL by an NbS radical induced a 10-fold increase in K_m, whereas V_{max} was unaffected [9]. Lipase regenerated by thiols from the above NbS-PPL had exactly the same kinetic parameters as the native enzyme. From these kinetic data and taking into account the fact that their actual significance is not clearly established, it was hypothesized that the accessible SHI in PPL was not essential for catalysis but was at, or near, the site involved in the attachment of PPL to hydrophobic interfaces [9].

The situation seems to be very different in the case of RGL. We have shown in this study that this lipase is inhibited by classical sulfhydryl reagents such as NbS2, 4-PDS, NEM and PM concomitantly with the modification of one sulfhydryl group per enzyme molecule (see fig.1). It can thus be concluded that RGL possesses an 'essential' sulfhydryl group for the expression of its catalytic activity. These results are comparable to those obtained with HGL [7].

In the search for covalent and specific lipase inhibitors, classical sulfhydryl reagents may be used as RGL inhibitors. Furthermore, hydrophobic derivatives such as C12-NbS can rapidly stop lipolysis when the enzyme is already adsorbed and active on its triacylglycerol substrate, unlike the classical sulfhydryl reagents (see fig.3). Experiments are now in progress to establish whether the SH modification affects the interfacial binding or catalysis.

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