while the isotope effect on V/K is given by

$$^{D}(V/K) = \frac{^{D}k_5 + C_f + C_r{}^{D}K_{eq}}{1 + C_f + C_r} \quad (A24)$$

where

$$C_f = \{(k_5/k_4)(1 + [H]/K_1) + (k_5/k_4)[I] \times [[k_3(k_2 + k_{12})]/k_2k_{11}](1/L_{i2} + [H]/L_1L_{i1})\}/\{(1 + [H]/K_1) + [I](1/K_{i2} + [H]/K_1K_{i1})\} \quad (A25)$$

and $C_r = k_6/k_7$.

In the case of NC_{asym}, the expressions for the isotope effects are given as

$$^{D}V = \frac{^{D}k + C_{vf} + C_r(^{D}K_{eq})}{1 + C_{vf} + C_r} \quad (A26)$$

where

$$C_{vf} = 1 + \frac{k_5}{k_2} + \frac{k_5}{k_9} + [I](1/L_{i2} + [H]/L_1L_{i1}) - [I]\frac{k_5k_{11}k_2}{k_3k_9(k_2 + k_{12})}(1/K_{i2} + [H]/K_1K_{i1}) \quad (A27)$$

and $C_r = k_6/k_7$ while

$$^{D}(V/K) = \frac{^{D}k + k_5/k_4 + (k_6/k_7)^{D}K_{eq}}{1 + k_5/k_4 + k_6/k_7} \quad (A28)$$

References

Cha, S. (1968) *J. Biol. Chem. 243*, 820.
Dalziel, K., & Dickinson, F. M. (1966) *Biochem. J. 100*, 491.
King, E. L., & Altman, C. (1956) *J. Phys. Chem. 6*, 1375.

Interface-Mediated Inactivation of Pancreatic Lipase by a Water-Reactive Compound: 2-Sulfobenzoic Cyclic Anhydride†

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ABSTRACT: 2-Sulfobenzoic cyclic anhydride (SBA) rapidly and selectively inactivates porcine pancreatic lipase (PPL) *only* when added during the hydrolysis of an emulsified ester such as tributyrin or dodecyl acetate. The present data suggest that the inactivation of PPL occurs preferentially at the oil/water interface and not in the aqueous phase, since colipase and bile salt were found to adversely affect the inhibition process. Moreover, it is shown that at a molar ratio of SBA to pure PPL of 1, 40% of the lipase activity was already irreversibly lost. Complete inactivation was observed at SBA to pure PPL molar ratios of 120. A 60% inactivation occurred when 0.5 mol of ^{3}H-labeled SBA was attached per mole of PPL. The SBA-inactivated PPL competes for binding to the dodecyl acetate/water interface as efficiently as the native enzyme. Larger SBA concentrations are required when crude lipase preparations are used as well as with pure PPL in the presence of bile salts and colipase. Lipases were found to have variable sensitivites to SBA inactivation, depending on their origin. In the presence of bile salts and tributyrin at pH 6.0, human gastric lipase activity was not affected by the presence of a 10^6 molar excess of SBA.

Achieving specific and covalent inactivation of lipolytic enzymes is a difficult task, because of nonmutually exclusive processes such as interfacial denaturation, changes in "interfacial quality", and surface dilution phenomena (Verger & de Haas, 1976; Dennis, 1987). Furthermore, the interfacial enzyme binding and/or the catalytic turnover can be diversely affected by the presence of potential amphipatic inhibitors (Verger, 1984; Kurganov et al., 1985).

A large number of chemical reagents has been used for modifying several "essential" amino acid residues in porcine pancreatic lipase (Verger, 1984; Desnuelle, 1986). Pancreatic lipase is not inactivated by classical water-dispersed serine esterase inhibitors such as diisopropyl fluorophosphate, benzamidine, or phenylmethanesulfonyl fluoride; in fact, these serine protease inhibitors are currently used in a millimolar concentration range to prevent pancreatic lipase proteolysis from occurring during the purification procedures (Verger, 1984). However, some other hydrophobic aromatic sulfonyl halides such as 4-iodobenzenesulfonyl chloride and 1-(dimethylamino)naphthalene-5-sulfonyl chloride (dansyl chloride) inactivate porcine pancreatic lipase by reacting with several residues, probably including a serine (Verger, 1970; Maylié et al., 1972).

Desnuelle et al. (1960) first showed that gum arabic emulsified diethyl *p*-nitrophenyl phosphate irreversibly inactivates porcine pancreatic lipase, in sharp contrast with aqueous solutions of this organophosphorus compound. Maylié et al. (1972) and Rouard et al. (1978) described the covalent modification of a serine residue of pancreatic lipase induced by mixed micelles of diethyl *p*-nitrophenyl phosphate and bile salts. The finding that colipase played an essential role in this inactivation process confirms that the first step in the inactivation is an interaction between lipase and bile salt containing micelles. The requirements of lipase as far as specific sub-

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strates and inactivators are concerned therefore seem to be very similar. The stoichiometric and selective modification of Ser 152 (Guidoni et al., 1981) is one example in which a micellar solution of diethyl *p*-nitrophenyl phosphate was used to covalently modify an amino acid residue which was supposed to be involved in the interfacial lipase binding (Chapus & Sémériva, 1976).

Using bis(*p*-nitrophenyl) methylphosphonate (BNMP),[1] Sikk et al. (1985) completely and irreversibly inactivated pancreatic lipase activity on tributyrin emulsion whereas the activity of the enzyme on *p*-nitrophenyl acetate solution remained unchanged. As previously observed (Chapus & Sémériva, 1976) in the case of the diethyl *p*-nitrophenyl phosphate treated lipase, the BNMP-modified enzyme did not bind onto silicon-coated glass beads (Sikk et al., 1985). The same authors (Sikk et al., 1986), using increasing amounts of [2,3-^{3}H]succinic anhydride, revealed the presence of two reactive amino groups in the enzyme. One of these groups was identified as the N-terminal α-amino group of the enzyme and found to be essential for the colipase-dependent activity of pancreatic lipase to occur, as previously suggested (Erlanson, 1977). More recently, Hadvary et al. (1988) and Borgström (1988) described the covalent and selective inhibition of pancreatic lipases from several species, including man, by tetrahydrolipstatin. The lipase is progressively inactivated though the formation of a long-lived covalent intermediate, probably with a 1:1 stoichiometry. Triolein and a boronic acid derivative retard the rate of inactivation.

The aim of the present research was to test a number of hydrophobic organic compounds suspected of being lipase inactivators. 2-Sulfobenzoic cyclic anhydride, a known lysine reagent, was selected and studied as a water-reactive and interface-mediated inactivator prototype.

Materials and Methods

Enzymes. Porcine pancreatic lipase (PPL) was prepared from pancreas (Verger, 1984) in a form saturated with colipase; specific activity at 37 °C on a tributyrin emulsion as 4000 units/mg. Human gastric lipase (HGL) (Tiruppathi & Balasubramanian, 1982) was purified from gastric juice (a generous gift from Professor H. Sarles, Hopital Sainte-Marguerite, 13009 Marseille, France); specific activity at 37 °C on a tributyrin emulsion was 1200 units/mg. Rat lingual lipase (RLL) (Blangero, 1982; Field & Scow, 1982) had a specific activity of 200 units/mg. These mammalian lipases were purified at the laboratory according to previously published methods. Microbial lipases from *Rhizopus delemar* and *Rhizopus arrhizus* were also purified at the laboratory by Dr. A. Sugihara, as described (Iwai & Tsujisaka, 1974). A supernatant of a *Staphylococus epidermidis* culture containing lipase activity (Pablo et al., 1974) was a gift from Dr. J. M. Ballester (GERME S.A. Co., 27, Boulevard Charles-Moretti, 13014 Marseille, France). Pure porcine pancreatic phospholipase A_2 was a gift from Dr. G. H. de Haas (Laboratory of Biochemistry, Utrecht, The Netherlands). Bovine trypsin and chymotrypsin were from Sigma (St. Louis, MO).

Chemicals and Inhibitors. Taurodeoxycholic acid sodium salt (NaTDC) was purchased from Sigma. *N*-Benzoyl-L-arginine ethyl ester (BAEE), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), dodecyl acetate, succinic anhydride, phthalic anhydride, methanesulfonyl chloride, 4-iodobenzenesulfonyl chloride, and 2-sulfobenzoic cyclic anhydride (SBA) were from Aldrich (Strasbourg, France). 2-Sulfobenzoic acid imide sodium salt, *p*-nitrophenyl acetate (PNPA), *p*-toluenesulfonyl chloride, and tributyrylglycerol were from Fluka (Buchs, Switzerland). 3*H*-1,2-Benzoxathiole 2,2-dioxide was purchased from Eastman Organic Chemicals. α-Chloro-α-hydroxytoluenesulfonic acid sultone was from Jansen Chimica. ^{3}*H*-1,2-Benzoxathiole 1,1-dioxide was synthesized as described by Heidema and Kaiser (1968). 1-Chlorotridecan-2-one and 1-chlorononadec-10-en-2-one were a gift from Professor B. Entressangles (Université de Bordeaux, France).

Bromoacetates of hexanol, heptanol, dodecanol, and tetradecanol were prepared by carrying out a reaction between an equimolar amount of bromoacetyl chloride (Fluka) and the corresponding aliphatic alcohol in anhydrous hexane, under a nitrogen stream. These bromoacetyl esters were purified by silica gel chromatography. Glyceride analogues were prepared as follows: esterification of glycerol by an equimolar amount of decanoyl chloride (Fluka) led to a mixture of tri-, di-, and monodecanoates, which was separated by preparative silica gel chromatography. the subsequent action of bromoacetyl chloride on 1,3-didecanoylglycerol and *rac*-1-decanoylglycerol, under the same conditions as above, afforded the required products, which were finally purified by silica gel chromatography. Sulfopropanoic acid cyclic anhydride was synthesized as described by Kharasch and Brown (1940). Monodecanoyl-, monododecanoyl-, and monohexadecanoylglycidols were synthesized by glycidol acylation by using the corresponding acyl chloride according to Elzant (1987).

^{3}H-Labeled SBA was prepared upon request by the Commissariat à l'Energie Atomique (Service des Molécules Marquées, CEA, Saclay, France). The tritiation reaction was performed under magnetic stirring, in a 5-mL flask containing 20 mg of tetrabromo-2-sulfobenzoic cyclic anhydride (Aldrich Chemical Co.) and 380 mg of SBA as carrier dissolved in 2 mL of anhydrous peroxide-free tetrahydrofuran (THF) with 100 mg of 10% palladium on charcoal as catalyst. the reduction reaction was run during 10 min with 10 mL of gaseous hydrogen containing 25 Ci of $^3H_2$. About 3 mL of the tritium-labeled gas was used during the reduction reaction. The reaction medium was then filtered, the solvent was evaporated under vacuum, and the residue was redissolved in 2 mL of anhydrous THF. A sample of 20 μL of this stock solution was found to contain 40 mCi of ^{3}H-labeled SBA. The radiolabeled SBA was stored in dry form at the CEA (Saclay, France). Small samples of ^{3}H-labeled SBA were further purified by sublimation, under a vacuum (0.1 mmHg) at 80–100 °C, of a mixture containing 1 mCi of ^{3}H-labeled SBA and 40 mg of nonradiolabeled SBA. Stock solutions of ^{3}H-labeled SBA (2.4 mg/mL with a specific radioactivity of 9×10^9 dpm/mmol) were prepared before use in dry ethyl acetate.

Methods. Protein concentrations were determined according to Lowry's method (Lowry et al., 1951) or by spectrophotometric means at 280 nm with the following absorption coefficients, E (1%, 1 cm): mammalian and microbial lipases, 13.5; trypsin and chymotrypsin, 20; phospholipase A_2, 14.

Lipase activities were determined with mechanically stirred emulsions of tributyrylglycerol (0.5 mL) or dodecyl acetate (0.5 mL) as substrate in the presence of NaTDC (3 mM final concentration) in 10 mL of NaCl, 0.15 M. Lipase activity was monitored titrimetrically at pH 8.0 (pancreatic lipase) or pH 6.0 (gastric lipase) with a pH-stat (TTT 80, Radiometer,

[1] Abbreviations: SBA, 2-sulfobenzoic cyclic anhydride; THF, tetrahydrofuran; E-600, diethyl *p*-nitrophenyl phosphate; BNMP, bis(*p*-nitrophenyl) methylphosphonate; PPL, porcine pancreatic lipase; HGL, human gastric lipase; RLL, rat lingual lipase; RGL, rabbit gastric lipase; BAEE, *N*-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; PNPA, *p*-nitrophenyl acetate; BSA, bovine serum albumin; I/E, inhibitor to enzyme molar ratio; NaTDC, taurodeoxycholic acid sodium salt.

Copenhagen, Denmark) equipped with a thermostated (37 °C) vial. In all the assays, the lipase activities were derived from the initial slopes of the kinetics and expressed as international units. One international lipase unit corresponds to 1 μmol of fatty acid liberated per minute. Trypsin and chymotrypsin activities were determined with BAEE and BTEE (1 mM final concentration), respectively, as substrates, and hydrolysis was monitored titrimetrically at pH 7.8 and 37 °C (Guy et al., 1966). Phospholipase A_2 activity was determined with emulsified egg yolk lipoproteins as substrate (Nieuwenhuizen et al., 1974), and hydrolysis was monitored titrimetrically at pH 8.0, 37 °C.

Methods of Testing for Lipase Inactivation. Two methods, which are depicted schematically in Figure 1, were used in order to determine the capacity of a potential lipase inactivator.

Method A. This method was set up in order to test whether a direct reaction occurred in aqueous medium between lipase and the organic compound to be tested, in the absence of substrate. Lipase (10 nM final concentration) was injected into the thermostated (37 °C) reaction vessel of a pH-stat containing 10 mL of NaCl (0.15 M), with vigorous stirring. Two minutes later, the reagent to be tested was added to a final concentration of either 1 mM [inhibitor to enzyme molar ratio of (I/E) 10^5] or 2 μM (I/E = 200) from a stock solution (1 mg/mL) in anhydrous ethyl acetate. Two minutes later the lipase substrate, 0.5 mL of tributyrylglycerol or dodecyl acetate, was added, and residual enzyme activity was measured at pH 8.0 (pancreatic lipase) or pH 6.0 (gastric lipase).

Method B. This method was designed to test whether any reaction occurred between lipase and the potential inactivator in the presence of a water-insoluble substrate. Lipase (10 nM final concentration) was injected into the thermostated (37 °C) reaction vessel of the Radiometer pH-stat, containing 10 mL of NaCl (0.15 M), with vigorous stirring. Two minutes later the lipase substrate, 0.5 mL of tributyrylglycerol or dodecyl acetate, was added, and the enzyme activity was recorded during 3 min at constant pH. The reagent to be tested was added to a final concentration of either 1 mM (I/E = 10^5) or 2 μM (I/E = 200) from a stock solution (1 mg/mL) in anhydrous ethyl acetate–tributyrylglycerol (1/9 v/v). With both methods the amount of substrate used was checked to give the maximal lipase activity.

Method of PPL Inactivation by ^{3}H-Labeled SBA. Lipase (2 μM final concentration) was injected into the thermostated (37 °C) reaction vessel of the Radiometer pH-stat, containing 10 mL of NaCl (0.15 M) and 1 mL of dodecyl acetate, with vigorous stirring. Lipase activity was recorded during 2 min at constant pH (8.0) with NaOH (0.1 M) as titrant. In each labeling experiment the [^{3}H]SBA stock solution was used (2.4 mg/mL in dry ethyl acetate with a specific radioactivity of 9×10^9 dpm/mmol). This radiolabeled SBA solution was added to the titration vessel to a final SBA concentration ranging from 1.4 μM (I/E = 0.7) to 1.6 mM (I/E = 800). The reaction medium was maintained for 10 min at a constant pH of 8.0 in order to allow complete hydrolysis of the excess SBA into water-soluble sulfobenzoic acid. The total reaction medium was then extensively dialyzed three times against 1 L of Tris-HCl buffer, 0.1 M, pH 8.0. The contents of the dialysis bag were centrifuged for 30 min at 6500*g* in order to break the partly hydrolyzed dodecyl acetate emulsion. The lower water phase was concentrated to 1 mL and filtered through a Sephadex G-25 column equilibrated with a 10 mM phosphate buffer (pH 8.0) in order to eliminate any low molecular weight compounds. After gel filtration, the PPL concentration in the break-through fractions was evaluated according to Lowry's method (Lowry et al., 1951). The overall PPL recovered after the G-25 chromatography was found to be around 10% of the amount initially present in the incubation medium.

Results and Discussion

Preliminary Trials. All potential lipase inactivators were systematically tested with methods A and B. The choice of short incubation times at 37 °C under vigorous stirring at low PPL concentrations was due to the fact that PPL is sensitive to spontaneous denaturation. This preliminary study was inspired by the classical PA_2 inhibition achieved with *p*-bromophenacyl bromide (Volwerk et al., 1974). First, we turned our attention to compounds derived from fatty esters, aliphatic chloro ketones, and glyceride analogues bearing an electrophilic carbon atom. We hoped to obtain a lipid–enzyme complex which was active site directed and could generate a covalent intermediate after a nucleophilic attack on the electrophilic site of the potential inactivator. The existing compounds of this type were 1-chlorotridecan-2-one and 1-chlorononadec-10-en-2-one. The other compounds of this family were bromoacetyl derivatives of 1-hexanol, 1-heptanol, 1-dodecanol, 1-tetradecanol, and *p*-nitrophenol. The substrate analogues derived from glycerol were *rac*-1-decanoyl-2,3-bis(dibromoacetyl)glycerol, 2-(bromoacetyl)-1,3-didecanoylglycerol, 2-(bromoacetyl)-1,3-propanediol didecyl diether, 1,2-epoxypropyl decanoate, and 1,2-epoxypropyl hexadecanoate.

The aliphatic and water-insoluble fatty chloro ketones as well as the bromoacetyl derivatives of 1-alkanols used had no significant effect on lipase activity. Sometimes, in the presence of a finely dispersed emulsion of these compounds, a slow decrease in lipase activity was observed, probably due to interfacial enzyme denaturation. Neither the bromoacetyl derivatives of mono- or didecanoylglycerol nor the epoxy derivatives were retained as lipase inactivators. On the contrary, the inductive effect at the level of the ester linkage, brought about by the bromoacetyl moiety, actually increased the susceptibility to hydrolysis catalyzed by PPL. This effect has been extensively studied (Brockerhoff & Jensen, 1974).

The second family of compounds tested was chosen because of its known reactivity toward some nucleophilic side chains of amino acid residues. These compounds were succinic anhydride, phthalic anhydride, *p*-toluenesulfonyl chloride, methanesulfonyl chloride, 4-iodobenzenesulfonyl chloride, *p*-toluenesulfonic anhydride, *p*-toluenesulfonic benzoic anhydride, 3-sulfopropanoic acid cyclic anhydride, and 2-sulfobenzoic cyclic anhydride (SBA). Very few chemical compounds among those tested were able to significantly decrease PPL activity under the experimental conditions described in methods A and B. Chemical compounds that showed any inactivation properties were further tested at higher dilutions. At final concentrations equal to or lower than 2 μM (inactivator to lipase molar ratio of 200), only SBA was able to rapidly inhibit PPL activity and only when method B was used. The latter anhydride has been previously used for modifying ε-amino groups of the lysyl residues in proteins (Bagree et al., 1980).

In order to evaluate the minimal SBA molecule structural requirements for PPL to be inactivated, we checked five chemical analogues of SBA: 3*H*-2,1-benzoxathiole 1,1-dioxide, which is known to be a chymotrypsin inhibitor (Heidema & Kaiser, 1968), 3*H*-1,2-benzoxathiole 2,2-dioxide, 3-chloro-3*H*-1,2-benzoxathiole 1,1-dioxide, sulfopropanoic acid cyclic anydride, and 2-sulfobenzoic acid imide sodium salt. All these compounds belong to the cyclic sulfonic acid family. None

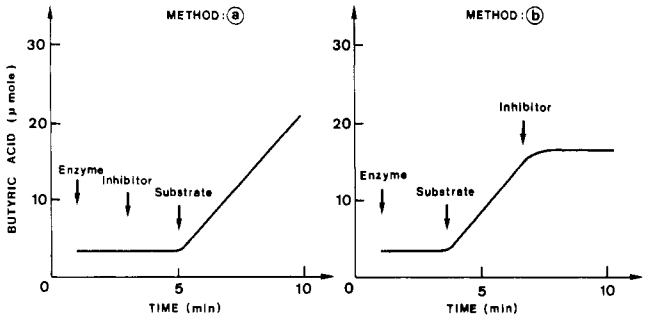

FIGURE 1: Methods of testing for lipase inactivation. Effects of SBA on PPL activity, according to method A or method B. Arrows indicate the successive injections of enzyme (0.1 nmol) of pure pancreatic lipase, inhibitor (20 nmol of SBA), and substrate (0.5 mL of tributyrin) into NaCl, 0.9%, 10-mL final volume. Method A: Inhibitor (SBA, 20 nmol) dissolved in 100 μL of anhydrous ethyl acetate. Method B: Inhibitor (SBA, 20 nmol) dissolved in 100 μL of anhydrous ethyl acetate/tributyrin (1/9 v/v).

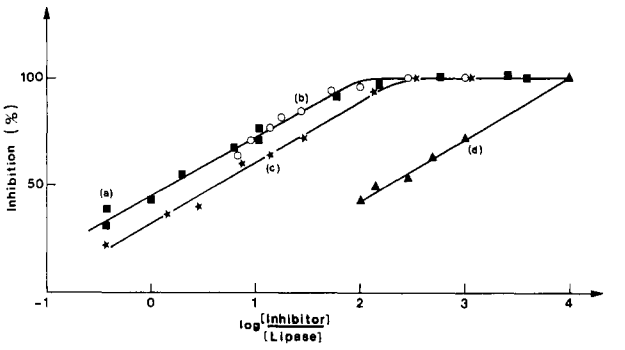

FIGURE 2: Influence of SBA concentration on PPL activity in the presence of various emulsions, according to method B. (Curve a) (■) Constant concentration of pure porcine pancreatic lipase (20 nM) and variable concentrations of SBA ranging from 8 nM to 100 μM. Substrate: tributyrin (0.5 mL) in NaCl, 0.9%, 10-mL final volume. (Curve b) (○) Constant concentration of SBA (1 μM) and variable concentrations of pure porcine pancreatic lipase ranging from 1 to 160 nM. Substrate: tributyrin (0.5 mL) in NaCl, 0.9%, 10-mL final volume. (Curve c) (*) Constant concentration of pure human pancreatic lipase (20 nM) and variable concentrations of SBA ranging from 8 nM to 20 μM. Substrate: dodecyl acetate (0.5 mL) in NaCl, 0.9% 10-mL final volume. (Curve d) (▲) Constant concentration of pure pancreatic lipase (saturated with colipase) (20 nM) and variable concentrations of SBA ranging from 2 to 200 μM, in the presence of NaTDC, 3 mM. Substrate: tributyrin (0.5 mL) in NaCl, 0.9%, 10-mL final volume.

of them was found to inactivate PPL when tested with method A or method B.

The choice of method is critical if the SBA inactivation capacity is to be determined (see Figure 1). This important statement can be explained qualitatively in terms of the fact that SBA is at the same time lipophilic and highly water reactive. Total hydrolysis at 2 mM and pH 8.0 in aqueous medium takes around 10 s, judging from the proton release under pH-stat conditions. This lifetime was increased up to 30 s in the presence of tributyrylglycerol or dodecyl acetate. This high reactivity with water makes surface behavior studies very difficult to carry out at lipid/water interfaces. The lipase inactivation capacity of SBA is therefore limited by its short lifetime in aqueous medium. In the presence of a tributyrylglycerol emulsion the lipid-solubilized SBA will have an increased half-life and probably an increased interfacial concentration. Both factors will enhance SBA reactivity with adsorbed lipase molecules. In the absence of an interface, i.e., in pure aqueous medium (method A), the water molecules (55 M) compete efficiently for SBA hydrolysis. The diacid form generated by SBA hydrolysis is unreactive toward lipase. Method B was used exclusively throughout the rest of the present study. With this method, we checked that identical PPL inactivation occurred when the anhydride was injected after variable tributyrylglycerol or dodecyl acetate hydrolysis periods (1–15 min). Under these experimental conditions, the reaction products formed did not interfere with the inactivation process. Inactivation by SBA, with method B, was not reversed by the subsequent addition of hydroxylamine (data not shown).

Influence of SBA Concentration on PPL Activity in the Presence of Various Emulsions. With method B, as shown in Figure 2, increasing the molar ration of SBA to PPL resulted in a progressive lipase inactivation. Curves a and b in Figure 2, depicting this dependence, are superimposable whether PPL or SBA concentration was used. A 50% PPL (20 nM) inactivation was observed at an SBA concentration of 30 nM whereas the lipase activity was entirely lost when the SBA concentration was increased to 2 μM. As expected from the comparison between the results obtained with methods A and B, the inactivation capacity of SBA depends very much upon the "interfacial quality" of the system used. At pH 8.0, PPL hydrolyzes dodecyl acetate with a specific activity corresponding to 1% of that measured with tributyrylglycerol. With both emulsions, saturation conditions were reached by using an excess lipid, making all lipase interfacially adsorbed. SBA inactivation in the presence of dodecyl acetate was less pronounced than that when tributyrylglycerol was used (Figure 2, curve c): 20 nM lipase was inactivated to 50% at an SBA concentration of 80 nM. This difference in inactivation level in the presence of a tributyrylglycerol vs dodecyl acetate emulsion may be attributable to the variable solubility of SBA in these esters or to a difference in SBA and/or lipase orientation at the ester/water interface.

At 3 mM NaTDC with tributyrylglycerol emulsion, 50% inactivation of PPL was observed at 0.2 mM SBA (Figure 2, curve d). Lipase inactivation induced by SBA was considerably reduced, probably due to nonspecific competitive reactions of SBA with bile salts. In that case NaTDC was probably adsorbed at the oil/water interface and may have competed with PPL for SBA, or NaTDC micelles may have dissolved a fraction of SBA and thus reduced the effective SBA concentration available for PPL inactivation at the interface. As an overall consequence, proteins and bile salts will have reduced the lipase inactivation capacity of SBA. We checked that SBA-inactivated PPL was still able to hydrolyze the water-soluble *p*-nitrophenyl acetate under standard assay conditions (data not shown). However, it is doubtful whether *p*-nitrophyl acetate hydrolysis faithfully reflects the functionality of the pancreatic lipase active site (Verger, 1984).

Influence of Colipase in the Presence of Bile Salts on PPL Inactivation by SBA. It is generally accepted that increasing concentrations of NaTDC desorb pure PPL from the oil/water interface and thus increase the concentration of free PPL in the water phase (Verger, 1984; Borgström, 1984; Desnuelle, 1986).

In order to ascertain the interfacial nature of PPL inactivation by SBA, we took advantage of the well-established role of colipase in the interfacial anchoring of PPL in the presence of supramicellar concentrations of bile salts (Verger, 1984; Borgström, 1984; Desnuelle, 1986). The results of these experiments are presented in Figure 3. When the NaTDC concentration was increased, pure PPL (containing no colipase) was desorbed from the tributyrylglycerol interface, which explains the decrease in the slopes of the four kinetic experiments after enzyme injection. In all four cases, SBA injection completely and instantaneously abolished the expressed PPL

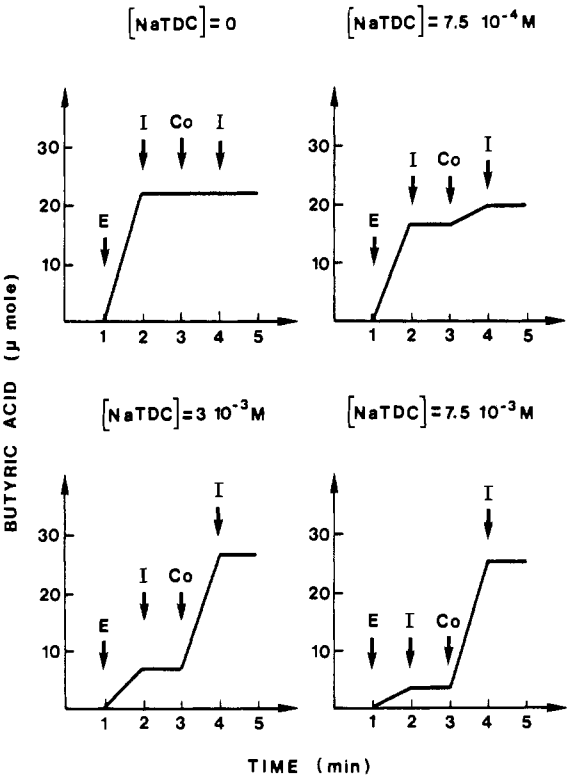

FIGURE 3: Influence of colipase and bile salts on PPL inactivation by SBA. Arrows indicate successive injections of (E) pure porcine pancreatic lipase (0.1 nmol), (I) SBA (2 μmol), and (Co) colipase (saturating amounts). Substrate: tributyrylglycerol (0.5 mL) in NaCl, 0.9%, 10-mL final volume.

activity. Under these conditions, a later colipase injection had a selective effect, restoring more lipase activity in those cases where more NaTDC was initially present. It can be seen from the kinetics presented in Figure 3 that the sum of the PPL activity measured immediately after lipase injection and that measured after colipase injection was constant and equal to the value measured when no SBA was added. These kinetic experiments strongly support our previous interpretation that SBA inactivates only PPL molecules that are adsorbed at the surface of the emulsion and not the free enzyme in aqueous medium. This may be attributable to the increased half-life and increased interfacial concentration of SBA in the presence of a tributyrylglycerol emulsion.

PPL Inactivation and Radiolabeling by [^{3}H]SBA. Figure 4 gives the data obtained with ^{3}H-labeled SBA during PPL inactivation in the presence of a dodecyl acetate emulsion, as described under Materials and Methods. Since dodecyl acetate was hydrolyzed at 1% of the corresponding rate observed when tributyrylglycerol was used as substrate, we selected the poorer substrate in order to be able to use higher lipase concentrations and hence obtain a substantial radiolabeling with [^{3}H]SBA. As previously observed with unlabeled SBA, complete PPL inactivation occurred when a molar ratio of SBA to PPL close to 120 was used. Under these conditions a 1:1 stoichiometric labeling of PPL by [^{3}H]SBA occurs. Furthermore, a linear relationship was found to exist as regards the number of SBA residues attached per PPL molecule when lower molar ratios of anhydride to enzyme were used. However, when this ratio was 800, 3.5 SBA residues were bound per lipase molecule. The latter result is in agreement with the known capacity of SBA to react covalently with several side-chain amino acid residues in proteins. However, as illustrated in the insert in Figure 4 at low SBA molar ratios, a continuous hyperbolic relationship was found to exist between the degree of PPL

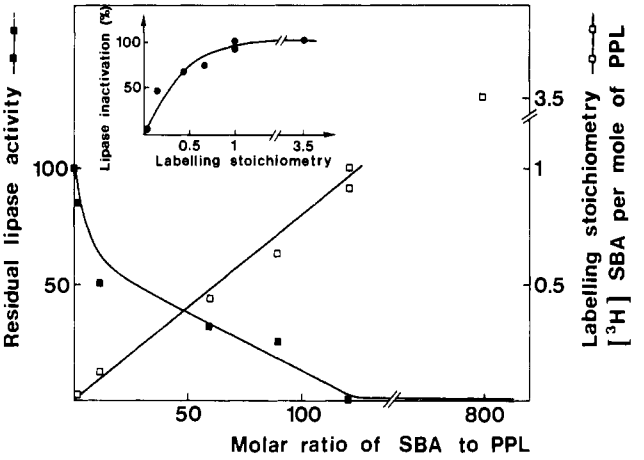

FIGURE 4: PPL inactivation and radiolabeling by [^{3}H]SBA in the presence of a dodecyl acetate emulsion: (■) residual lipase activity; (□) labeling stoichiometry as ^{3}H-labeled SBA per mole of PPL. (Insert) Inactivation: radiolabeling relationship during PPL inactivation by [^{3}H]SBA; data replotted from main figure. See Materials and Methods for experimental details.

Table I: Competition for Interfacial Binding between Native and SBA-Inactivated PPL[a]

native PPL (μg)	SBA-inactivated PPL (μg)	molar fraction of native lipase (%)	remaining lipase activity (%)
640	8	98	91
640	44	93	77
640	88	88	66
640	450	58	56
640	660	49	46

[a] PPL (640 μg) was injected into the thermostated (37 °C) reaction of the Radiometer pH-stat, containing 10 mL of NaCl (0.15 M) and 0.1 mL of dodecyl acetate, with vigorous stirring. Lipase activity was recorded during 2 min at constant pH 8.0 with NaOH (0.1 M) as titrant. Variable amounts of SBA-inactivated PPL, ranging from 8 to 660 μg, were then injected. Under these experimental conditions, we independently determined the maximal amount of PPL which saturates the dodecyl acetate/water interface, since a further lipase addition will not significantly increase the hydrolysis rate. Furthermore, SBA addition (120 molar excess relative to the enzyme) inactivates all the lipase (640 μg) present in the assay. The latter point was checked by adding tributyrylglycerol, which resulted in no detectable residual enzyme activity.

inactivation and the labeling stoichiometry. For instance, 60% inactivation occurred when 0.5 mol of [^{3}H]SBA was attached per mole of PPL. When diethyl *p*-nitrophenyl phosphate (E-600) in micellar solution reacted with PPL, as described by Rouart et al. (1978), the resulting freshly prepared (diethylphosphoryl)lipase still reacted nearly stoichiometrically (0.8 mol/mol) with ^{3}H-labeled SBA (I/E = 132). These data indicate that serine 152 in PPL is not the amino acid residue involved in SBA inactivation.

Competition for Interfacial Binding between Native and SBA-Inactivated PPL. In order to check whether the SBA-inactivated PPL was still able to bind to the lipid/water interface, we conducted competition experiments between native and SBA-inactivated PPL by using a dodecyl acetate emulsion, which was selected because of its low rate of hydrolysis by the active enzyme. In each competition experiment, during dodecyl acetate hydrolysis, we used a fixed (640 μg) amount of PPL and variable amounts of SBA-inactivated PPL. The latter inactivated enzyme was from a 0.88 mg/mL stock solution prepared as described under Materials and Methods and ranged from 8 to 660 μg. Table I gives the remaining lipase activity as a function of the molar fraction of active lipase in

Table II: Effect of SBA on Various Esterases in the Presence of Emulsified Tributyrin, Using Method B[a]

enzymes	NaTDC (3 mM)	% inactivation
porcine pancreatic lipase[b]	+	100
porcine pancreatic lipase	−	100
human pancreatic lipase	+	100
human pancreatic lipase	−	100
porcine pancreatic juice	+	100
porcine pancreatic juice	−	65
pancreatic phospholipase A_2[c]	−	0
trypsin	−	0
chymotrypsin	−	0
horse liver esterase	−	0
human gastric lipase	+	0
human gastric lipase	−	0
human gastric juice	−	0
rabbit gastric lipase	+	0
rat lingual lipase	−	0
S. epidermidis lipase[d]	−	100
R. delemar lipase	−	100
R. arrhizus lipase	−	100

[a] Results obtained with method B with an inhibitor to lipase ratio of 200 in the absence of NaTDC and of 20 000 in the presence of NaTDC. [b] Purified enzyme of porcine pancreatic powder extract. [c] Tested also by Professor G. H. de Haas (Utrecht, The Netherlands) on egg yolk lecithin and dioctanoyllecithin as substrates in the presence or absence of tributyrin. [d] Supernatant from *S. epidermidis* broth.

the reaction medium. From these data it is clear that SBA-inactivated PPL competes for binding the dodecyl acetate/water interface as efficiently as the native enzyme. For instance, when equal amounts of native and SBA-inactivated PPL are simultaneously present, we measured a hydrolysis rate equal to 46% of the maximal activity obtained with the native lipase. We checked that the same results were obtained whether the native or SBA-inactivated PPL was added first to the assay system (data not shown). It is thus probable that the lipid binding domain of PPL is not greatly affected by SBA treatment. From these indirect evidences, we can tentatively conclude that SBA covalently inactivates PPL by modifying some essential amino acid residue (probably a lysine) present at or near the enzyme active center.

Effects of SBA on Various Hydrolases in the Presence of Emulsified Tributyrin. By use of method B, the inactivating effect of SBA was tested with other mammalian and microbial esterases of various origins. The results are summarized in Table II. Purified human pancreatic lipase or pure PPL, crude porcine pancreatic extracts, or pancreatic juice and *R. delemar*, *R. arrhizus*, and *S. epidermidis* lipases were inactivated after SBA injection. On the other hand, neither human gastric lipase, purified or present in total human gastric juice, nor lingual lipase was inactivated by SBA under identical experimental conditions. Three other pancreatic esterases, namely, trypsin, chymotrypsin, and phospholipase A_2, were insensitive to SBA. A quite interesting but different situation was described by Volwerk et al. (1974), who studied the classical pancreatic phospholipase A_2 inactivation by *p*-bromophenacyl bromide in the presence of lipid. Phospholipase A_2, as well as its zymogen, was effectively protected by substrate analogues when present in the monomeric state. In contrast, the inactivation rate of the active enzyme was enhanced when phospholipids were used in a concentration range above the critical micellar concentration, whereas the rate of the zymogen inactivation was not influenced by the presence of a phospholipid/water interface. The situation seems to be more paradoxical in the case of pancreatic lipase inactivation by SBA, where the presence of an emulsified substrate is required. It is worth mentioning that a new lipophilic sulfhydryl reagent recently synthetized (Gargouri et al., 1988) instantaneously inactivated gastric lipases (sulfhydryl enzymes) in the presence of a triglyceride emulsion.

From all the data presented in this paper we can conclude that, despite its apparently rather nonspecific chemical reactivity for the ϵ-amino group of the lysyl residues in proteins (Bagree et al., 1980), SBA selectively inactivates some lipases, but only in the presence of an emulsified substrate. This paradoxical situation can be explained in terms of the fact that SBA is both a lipophilic and a water-reactive compound. These two apparently antagonistic physicochemical properties give rise to a "transiently poisoned interface" capable of selectively inactivating some lipases. The local SBA concentration and orientation at the oil/water interface can be said to be topologically favorable to site-directed lipase inactivation. The nature of the amino acid side chain residues involved in the active site of lipases probably explains the selectivity observed since only some lipases were inactivated. The use of SBA as described under method B can be said to constitute a new type of interfacial inactivation procedure.

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Registry No. SBA, 81-08-3; [^{3}H]SBA, 121176-23-6; lipase, 9001-62-1; colipase, 55126-92-6; hexanol bromoacetate, 13048-32-3; heptanol bromoacetate, 18991-99-6; dodecanol bromoacetate, 3674-07-5; tetradecanol bromoacetate, 18992-01-3; hexanol, 111-27-3; heptanol, 111-70-6; dodecanol, 112-53-8; tetradecanol, 112-72-1; glycerol, 56-81-5; decanoyl chloride, 112-13-0; 1,3-didecanoylglycerol, 17598-93-5; *rac*-1-decanoylglycerol, 19670-50-9; 1,3-didecanoylglycerol bromoacetate, 121176-22-5; *rac*-1-decanoylglycerol bromoacetate, 121176-24-7; monodecanoylglycidol, 26411-50-7; monododecanoylglycidol, 1984-77-6; monohexadecanoylglycidol, 7501-44-2; dodecanoyl chloride, 112-16-3; hexadecanoyl chloride, 112-67-4; glycidol, 556-52-5.

REFERENCES

Bagree, A., Sharma, I. K., Gupta, K. C., Narang, C. K., Saund, A. K., & Mathur, N. K. (1980) *FEBS Lett. 120*, 275–277.

Blangero, C. (1982) Diplome d'Etudes Approfondies, CNRS, Marseille, France.

Borgström, B. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 151–183, Elsevier, Amsterdam.

Borgström, B. (1988) *Biochim. Biophys. Acta 962*, 308–316.

Brockerhoff, H., & Jensen, R. G. (1974) in *Lipolytic Enzymes*, pp 34–90, 275–277, Academic Press, New York.

Chapus, C., & Sémériva, M. (1976) *Biochemistry 15*, 4988–4991.

Dennis, E. A. (1987) *Drug Dev. Res. 10*, 205–220.

Desnuelle, P. (1986) in *Molecular and Cellular Basis of Digestion* (Desnuelle, P., Nören, A., & Sjöström, H., Eds.)

pp 275-296, Elsevier, Amsterdam.
Desnuelle, P., Sarda, L., & Ailhaud, G. (1960) *Biochim. Biophys. Acta 37*, 570-571.
Elzant, A. (1987) Thèse de Doctorat de Spécialité, Montpellier University, Montpellier, France.
Erlanson, C. (1977) *FEBS Lett. 84*, 79-82.
Field, R. B., & Scow, R. O. (1982) *J. Biol. Chem. 25*, 14563-14569.
Gargouri, Y., Moreau, H., Piéroni, G., & Verger, R. (1988) *J. Biol. Chem. 263*, 2159-2162.
Guidoni, A., Benkouka, F., De Caro, J., & Rovery, M. (1981) *Biochim. Biophys. Acta 660*, 148-150.
Guy, O., Grattecos, D., Rovery, M., & Desnuelle, P. (1966) *Biochim. Biophys. Acta 115*, 404-408.
Hadvary, P., Lengsfeld, H., & Wolfer, H. (1988) *Biochem. J. 256*, 357-361.
Heidema, J. H., & Kaiser, E. T. (1968) *J. Am. Chem. Soc. 90*, 1860-1866.
Iwai, M., & Tsujisaka, Y. (1974) *Agric. Biol. Chem. 38*, 1241-1247.
Kharasch, M. S., & Brown, H. C. (1940) *J. Am. Chem. Soc. 62*, 925-929.
Kurganov, B. I., Tsetlin, L. G., Malakhova, E. A., Chebotareva, N. A., Lankin, V. Z., Glebova, G. D., Berezovsky, V. M., Levashov, A. V., & Martinek, K. J. (1985) *J. Biochem. Biophys. Methods 11*, 177-184.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, J. R. (1951) *J. Biol. Chem. 193*, 265-275.
Maylié, M. F., Charles, M., & Desnuelle, P. (1972) *Biochim. Biophys. Acta 276*, 162-175.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32*, 147-154.
Pablo, G., Hammons, A., Bradley, S., & Fulton, J. E. (1974) *J. Invest. Dermatol. 63*, 231-238.
Rouard, M., Sari, H., Nurit, S., Entressangles, B., & Desnuelle, P. (1978) *Biochim. Biophys. Acta 530*, 227-235.
Sikk, P. F., Osa, A. V., & Aaviksaar, A. A. (1985) *FEBS Lett. 184*, 193-196.
Sikk, P. F., Osa, A. V., Lookene, A. G., & Aaviksaar, A. A. (1986) *Bioorg. Khim. 12*, 985-987.
Tiruppathi, C., & Balasubramanian, K. A. (1982) *Biochim. Biophys. Acta 712*, 692-697.
Verger, R. (1970) Thesis, Faculté des Sciences, Université de Provence, Marseille, France.
Verger, R. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Ed.) pp 83-150, Elsevier, Amsterdam.
Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77-117.
Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446-1454.

p-Nitrophenyl 3-Diazopyruvate and Diazopyruvamides, a New Family of Photoactivatable Cross-Linking Bioprobes†

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ABSTRACT: *p*-Nitrophenyl 3-diazopyruvate (DAPpNP) has been developed as a heterobifunctional cross-linking agent for synthesis of photoaffinity probes and photoactivatable cross-linking agents that are nucleophile specific. *p*-Nitrophenyl chloroglyoxylate is formed in high yield from oxalyl chloride and *p*-nitrophenol. Subsequent reaction with diazomethane produces DAPpNP in 50–60% overall yield. DAPpNP acylates primary and secondary amines to form 3-diazopyruvamides in high yields. 3-Diazopyruvamide derivatives have been formed from a wide variety of amines including aromatic amines, amino acids, and peptides. 3-Diazopyruvamides undergo photolysis and Wolff rearrangement at 300 nm to produce a ketene amide, which efficiently acylates nucleophilic species to form malonic acid amide derivatives. A family of photoactivatable 3-diazopyruvamide cross-linking agents was synthesized from amino acids. A cleavable, thiol-specific photoactivatable cross-linking agent was synthesized from cystamine. These reagents were caused to react with rabbit muscle aldolase to form mainly dimeric cross-linked species.

The techniques of photoaffinity labeling and the introduction of photogenerated cross-links in biological structures and aggregates are powerful methods for determining a number of characteristics of biochemical systems. These characteristics include the location and nature of the active site of enzymes, the receptors for hormones and drugs, the relationships of proteins within aggregate structures, and the study of protein–protein interactions involved in immunochemical interactions (Bayley & Knowles, 1977; Bayley, 1983; Chowdry & Westheimer, 1979; Tometsko & Richards, 1980). The development of new classes of photoactivatable reagents aids the elucidation of the structure of biological molecules by providing probes that can be fine tuned to react efficiently with a given biologically active target. Nevertheless, the number and variety of photosensitive functional groups for incorporation in these reagents is quite limited, and of these only the nitroaryl azides seem to have much popularity with the biochemical community (Westheimer, 1980; Bayley & Staros, 1984). The

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