Biochimicat et Biophysica Acta, 327 (1973) 101–113

BBA 67068

THE ROLE OF CARBOXYL GROUPS IN THE ACTIVITY OF PANCREATIC LIPASE

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(Received July 30th, 1973)

SUMMARY

The role of carboxyl groups in lipase activity was investigated by reaction with carbodiimides in the presence of a nucleophile. Several observations strongly suggested that this reaction was specific for carboxyls in the case of lipase (triacylglycerol lipase, EC 3.1.1.3).

With the *N*-ethyl-*N'*-(3-dimethylamino propyl) carbodiimide (EDC) and glycine ethyl ester, 14 carboxyls out of 51 were modified. This number was reduced to 5 when more bulky reagents (1-cylcohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide *p*-toluene sulfonate (CMC) and norleucine methyl ester) were used. In both cases, activity of the enzyme towards emulsified substrates was entirely suppressed. But the modified molecule still contained a catalytic site titratable with diethyl-*p*-nitrophenyl phosphate.

This observation taken in conjunction with others led to the assumption that the role of the carboxyl in lipase is structural rather than catalytic and that the active form of the enzyme resulting from adsorption at an hydrophobic interface rather than the native form is stabilized by the carboxyl.

INTRODUCTION

Pancreatic lipase (triacylglycerol lipase, EC 3.1.1.3) is a special carboxyl esterase acting very fast on aggregated substrate molecules[1]. If a number of studies have been devoted in recent years to the specificity of this enzyme (for a review, see ref. 1), little is known so far on the relationships between its chemical structure and catalytic properties. In contrast with earlier claims, however, the porcine enzyme has been shown not to be a sulfhydryl enzyme[2], but to contain most probably an essential serine residue reacting with diethyl-*p*-nitrophenylphosphate in the presence of bile salts[3]. By analogy with other carboxyl esterases and proteases, this residue has been considered to be the acylation site during the nucleophilic attack of the substrate[3].

Abbreviations: EDC, *N*-ethyl-*N'*-(3-dimethylamino propyl) carbodiimide; CMC, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide *p*-toluene sulfonate.

Moreover, the participation of a histidine residue in the catalytic site of lipase has also been strongly suggested by activity–pH dependence curves and photooxidation assays[4].

It is now of great interest to evaluate the role of carboxyl groups in the activity of lipase. Chymotrypsin activity has recently been postulated to be under the control of an aspartyl residue (Asp 102), probably through a charge-relay system[5] increasing the nucleophilicity of the acylable serine (Ser 195). An essential carboxyl has also been identified in other serine enzymes of known structure[6,7] and to play a variety of roles in lysozyme[8], triosephosphate isomerase[9], trypsin[10], pepsin[11] and carboxypeptidase[12]. To investigate the carboxyls of lipase, we have used the method described by Hoare and Koshland[13] which consists of reacting the proteins with a carbodiimide followed by condensation with a nucleophile. In this way, 14 carboxyl groups out of a total of 51 were shown to react very fast in native lipase with the *N*-ethyl-*N'*-(3-dimethylamino propyl) carbodiimide (EDC) and glycine ethyl ester. With a more bulky carbodiimide and nucleophile, namely 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide *p*-toluene sulfonate (CMC) and norleucine methyl ester, the number of reacting carboxyls could be reduced to 5. In both cases, lipase activity towards long-chain and short-chain triglycerides was observed to be completely abolished, suggesting that at least one carboxyl was essential. The role of this group, however, was considered to be structural rather than catalytic, since a fully functional catalytic site titratable with diethyl-*p*-nitrophenyl phosphate could be shown to exist in the inactive lipase derivative with 5 modified groups (5N-lipase).

MATERIAL AND METHODS

Material

The short-chain triglycerides (triacetin, tripropionin and tributyrin) were purchased from Fluka (Switzerland) and purified by previously described techniques[14]. The carbodiimides EDC and CMC, glycine ethyl ester and norleucine methyl ester hydrochloride were also obtained from Fluka but used without further purification. [^{14}C]Glycine ethyl ester hydrochloride (specific radioactivity, 18.5 Ci/mole) was purchased from the Commissariat à l'Energie Atomique (Saclay, France). Absence of free glycine in the preparations was checked by thin-layer chromatography on Silica gel G with a phenol–water (80:20, v/v) mixture as the eluent.

The organophosphate inhibitor diethyl-*p*-nitrophenyl phosphate was purchased from Thompson and Joseph Radlett (Great Britain) and recrystallized twice from chloroform. Cattle bile salts were prepared according to Benzonana and Desnuelle[15]. α-Glycerol monooctyl ether was synthesized in this Laboratory by Dr H. Sari (unpublished experiments). Urea (Prolabo, France) was recrystallized twice from ethanol.

Lipase

All assays were carried out with the unfractionated isoenzymes L_A and L_B purified from defatted porcine pancreas powder according to Verger *et al.*[16]. The preparations were free of protein contaminants except for a small amount of colipase strongly bound to the enzyme. An extinction coefficient ($E^{1\%}_{1\,cm}$) of 13.3 and a molecular weight value of 48 000 were used for the protein.

METHODS

Determination of lipase activity

This activity was routinely measured at pH 8.0 and 25 °C with an excess of purified tributyrin (0.5 ml) emulsified in 15 ml of 0.1 M NaCl. The release of butyric acid was followed and recorded with the aid of a Radiometer TTT 1a pH-stat. One lipase unit liberates 1 μmole of fatty acid per min under the conditions of the assays. Lipase was also determined in some cases with emulsions of long-chain triglycerides (commercial olive oil) at pH 9.0 and 25 °C in the presence of an optimal concentration of bile salts[17] or with micellar solutions of triacetin or tripropionin in 0.1 M NaCl at pH 8.0 (ref. 18).

Reaction of carbodiimides with lipase

Lipase (2–10 mg/ml) was dissolved at 22 °C in a 1 M labeled glycine ethyl ester hydrochloride solution (specific radioactivity, 1 Ci/mole) at pH 5.4. EDC was added to a final concentration of 62.5 mM and the pH was maintained constant by adding 0.1 M HCl in a pH-stat. The same procedure was employed with norleucine methyl ester and CMC, but the final concentrations of the reagents were 0.3 M and 30 mM, respectively. After predetermined time periods, the carbodiimide excess was destroyed by addition of 1 ml of 4.4 M sodium acetate at pH 5 and the remaining lipase activity was measured on an aliquot. Blank assays showed that lipase activity was stable under the experimental conditions employed and that, consequently, the observed decrease could be entirely attributed to the action of the reagents on the enzyme. Neither reactivation nor further loss of activity was noticed upon addition of the acetate buffer.

The modified enzyme was then precipitated from the bulk of the solution by 10% trichloroacetic acid and all the contaminants were removed by 5 washings with 5% trichloroacetic acid and absolute ethanol. Complete elimination of non-covalently bound glycine ethyl ester was checked by performing a blank with the labeled compound in the absence of carbodiimide. When soluble samples of the modified lipase were required, contaminants were removed by passage through a 1.2 cm × 60 cm Sephadex G-100 column in 0.1 M sodium acetate at pH 6.0. This passage revealed no polymeric forms of the enzyme, indicating the absence of intramolecular linkages during the reaction.

Amino acid analysis

The trichloroacetic acid precipitates were vacuum dried and hydrolyzed with 6 M tri-distilled HCl in sealed tubes for 24 h at 110 °C. Amino acid analysis were performed in duplicate in a Beckman automatic analyzer Model 120 B according to the usual procedure[19].

Radioactivity measurements

They were carried out with the aid of a Packard Tricarb scintillation spectrometer after dissolution of the samples in 10 ml of Bray's mixture.

Reaction of lipase with diethyl-p-nitrophenyl phosphate: active site titration and obtention of the diethylphosphoryl derivative of lipase

The method described for this purpose by Maylié *et al.*[3] was used in a slightly

modified form. Lipase solutions (about 3 mg/ml) in 0.1 M sodium acetate (pH 6.0) containing NaCl (0.1 M), $CaCl_2$ (50 mM) and bovine bile salts (0.3%) were incubated at room temperature with diethyl-*p*-nitrophenyl phosphate (final concentration, 4.6 mM). Inhibition was 93–99% after 3 h. The released *p*-nitrophenol was measured at 400 nm after dilution of 0.2 ml samples with 1 ml of a 0.5 M Tris–HCl buffer at pH 9.0. Absorbance was read at once against a blank without enzyme in order to take care of any spontaneous hydrolysis of diethyl-*p*-nitrophenyl phosphate. At the end of the incubation, lipase activity towards aggregated substrates had almost completely disappeared and 1 mole of *p*-nitrophenol per mole of enzyme could be measured. Therefore, these conditions appear to be very suitable for active site titration in lipase solutions.

For preparative purposes, the inactive diethylphosphoryl derivative of lipase was filtered through a 1.8 cm × 20 cm Sephadex G-25 column equilibrated and eluted with a 1 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl.

Obtention of a complex between lipase and micelles of α-glycerol monooctyl ether

Mixtures of lipase (8.8 mg/ml) and α-glycerol monooctyl ether (0.77 mg/ml) in 1 M NaCl at pH 5.2 were filtered according to Sari *et al.* (Sari, H., Entressangles, B. and Desnuelle, P., unpublished results) through Sephadex G-100 equilibrated and eluted with a pH 5.2 solution containing the same concentration of NaCl and α-mono-ether. The unretarded peak including about half the lipase bound to mono-ether micelles was collected separately.

Denaturation of lipase in urea

The kinetics of lipase denaturation in 8 M urea was followed at 22 °C by differential spectrophotometry at 294 nm in a Cary recording spectrophotometer Model 14. The first-order rate constant of denaturation was given by the slope of the linear plot $\log \Delta A_{max} - \Delta A_t / \Delta A_{max}$ *vs* time, in which ΔA_{max} and ΔA_t are the absorbance variations observed, respectively, after 90 min (maximal value) and at time t.

RESULTS

Total number of carboxyl groups in denatured lipase

Lipase solutions (2 mg/ml) in 8 M urea at pH 5.6 were incubated for 30 min at room temperature to insure full denaturation of the enzyme. Then, EDC and unlabeled glycine ethyl ester were added, and incubation was pursued as indicated in Material and Methods. The amino acid composition of aliquots removed after 0, 10, 60 and 180 min was determined by the usual technique. Table I indicates that the glycine content of lipase, which did not initially exceed 39, rose to 90 during the incubation. No further increase was observed beyond 180 min. It may, therefore, be assumed that lipase contains approximately 51 free carboxyl groups whereas a total of 96 aspartic and glutamic acids are known to exist in the acid hydrolyzates of the enzyme[16]. This leaves about 45 for the amides (asparagine + glutamine).

Reaction of native lipase with EDC and glycine ethyl ester

When the native enzyme was employed, a more selective reaction involving "exposed" groups only could be expected to take place. The effect of carboxyl modi-

TABLE I

INCORPORATION OF GLYCINE INTO DENATURED LIPASE BY INCUBATION WITH EDC AND GLYCINE ETHYL ESTER

Only acidic and neutral amino acids were determined. Their number per mole of enzyme was calculated by reference to known values for alanine and phenylalanine, 20 and 23, respectively (ref. 16).

Amino acid	*Number of residues per mole of lipase after an incubation of (in min):*			
	0	*10*	*60*	*180*
Ala	19.9	20.2	20.1	20.2
Asp	58.7	59.7	58.8	58.7
½ Cys	12.0	11.3	10.2	11.0
Glu	38.1	38.0	38.8	38.0
Gly	38.9	66.2	87.0	90.3
Ile	23.2	22.8	22.6	22.4
Leu	27.0	27.9	27.6	27.6
Met	3.5	3.4	3.3	3.2
Phe	23.1	22.9	23.0	22.8
Pro	23.7	24.2	24.5	23.6
Ser	25.4	26.9	25.5	25.0
Thr	22.7	22.3	20.6	22.6
Tyr	14.7	14.4	13.2	11.6
Val	32.7	32.8	32.7	29.9

fication on the activity of the enzyme could also be evaluated. [^{14}C]-labelled glycine ethyl ester was utilized in these assays in order to follow glycine incorporation by both amino acid analysis and radioactivity counting.

The time course of this incorporation and lipase inactivation is illustrated by Figs 1 and 2. The 2 processes are seen to be first order with almost identical rate constants. Complete inactivation is observed in this case when 14 carboxyls are modified.

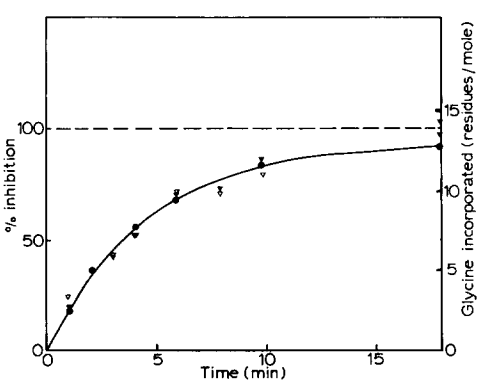

Fig. 1. Time course of glycine incorporation; ▲, radioactivity counting; △, amino acid analysis and lipase inactivation (●). The maximal number of 14 incorporated glycine was estimated by the method of Guggenheim[20].

Reaction of native lipase with CMC and norleucine methyl ester

The results presented in Fig. 1 are consistent with the view that one or several highly reactive carboxyl groups are involved in lipase activity. Considering that the

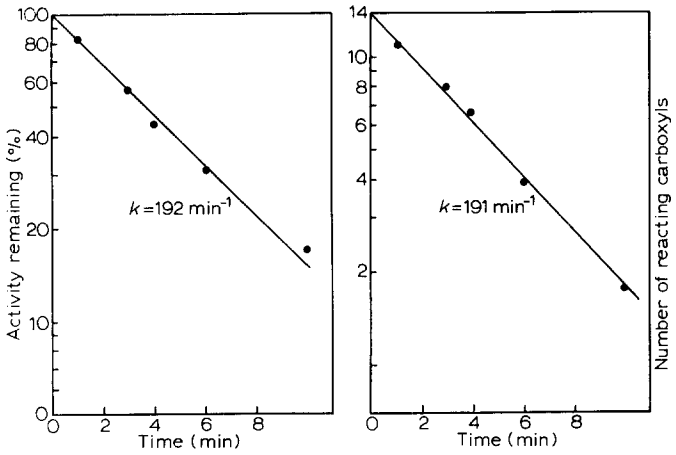

Fig. 2. Semi logarithmic plot of the variations *vs* time of enzyme activity (left) and number of carboxyl groups (right) during incubation of lipase with EDC and glycine ethyl ester.

14 modified groups possessed about the same reactivity, it appeared hardly possible to reduce their number by using either milder conditions for the incubation or lower concentrations of the reagents. By contrast, as shown by Figs 3 and 4, a more bulky carbodiimide and nucleophile, CMC and norleucine methyl ester, induced the modification of only 5 carboxyls. The pseudo-first-order rate constant of incorporation and inactivation were again observed to be very similar under these new conditions. The apparently well-defined lipase derivative thus obtained, in which 5 carboxyl groups are modified, will from now on be designated 5N-lipase.

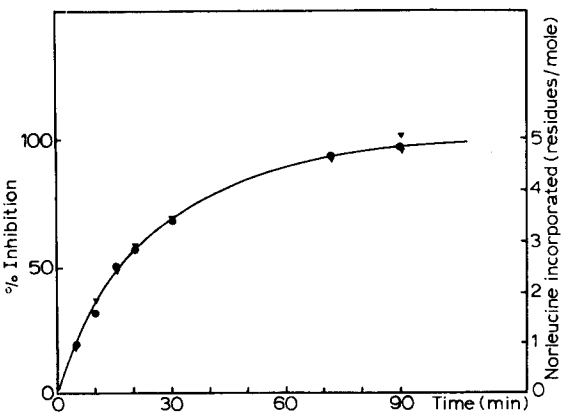

Fig. 3. Time course of norleucine incorporation; ▼, by amino acid analysis and of lipase inactivation (●). As indicated in Material and Methods, a 2-mg/ml solution of native lipase was reacted with 30 mM CMC and 30 mM norleucine methyl ester at pH 5.25. The value of 5 norleucine incorporated into the protein was as before derived from a Guggenheim plot[20].

Specificity of the reaction of carbodiimides with lipase

Although often used for the specific modification of carboxyls in proteins[10,13,21–23], carbodiimides may be expected to react with other nucleophilic groups of these

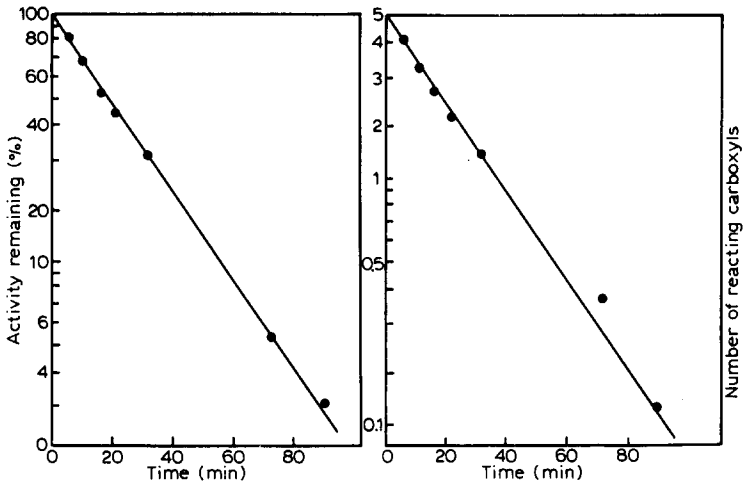

Fig. 4. Semi-logarithmic plot of the variations *vs* time of enzyme activity (left) and of the number of modified carboxyls (right) during the reaction of native lipase with CMC and norleucine methyl ester. Same experimental conditions as for Fig. 3. Gradient $k = 0.04\ min^{-1}$.

molecules[13,24–26], namely hydroxyls, imidazoles, amines and thiols. It was, therefore, of interest to study the specificity of the reaction in the peculiar case of lipase and of the 2 carbodiimides EDC and CMC.

The first assays in this respect showed that the pseudo-first-order rate constant of lipase inactivation was independent of the concentration of enzyme and nucleophile, but varied linearly with the concentration of the 2 carbodiimides in the whole range of their respective solubilities. This finding excluded the possibility to determine experimentally the dissociation constant of the enzyme–carbodiimide complex. It could, however, be deduced from existing data that this constant was higher than 100 mM. The conclusion was that, in sharp contrast with the observations reported in the case of chymotrypsin[27], carbodiimides appeared to display no especially large affinity for any peculiar group in lipase and, consequently, not to behave as an active site-directed reagent.

However, it was noteworthy that, as shown by Fig. 5, the rate constant of the inactivation reaction was strongly pH dependent. Investigations were limited here by the instability of lipase below pH 5. But, the shape of the curve reproduced in Fig. 5 strongly suggests that the pK of the group (or groups) involved in the reaction is lower than 5.5. This value is quite consistent with the assumption that carboxyls participate in the reaction leading with carbodiimides to inactivation.

When the various protein groups able to react with carbodiimides are now considered, amines can be at once disregarded because of their high pK which should preclude any reaction at pH 5.5 (ref. 28). With tyrosine and histidine, the products resulting from carbodiimide action have been reported to be stable during acid hydrolysis[24,28]. They should, therefore, cause a significant loss of both amino acids in the hydrolyzates of the modified lipase. Table II shows that no loss of these amino acids can be observed up to 92 % inactivation. Table II also indicates that the 2 SH groups known to exist in lipase[2] remain unaltered during the reaction.

Finally, a serine residue has been shown to react in chymotrypsin with carbo-

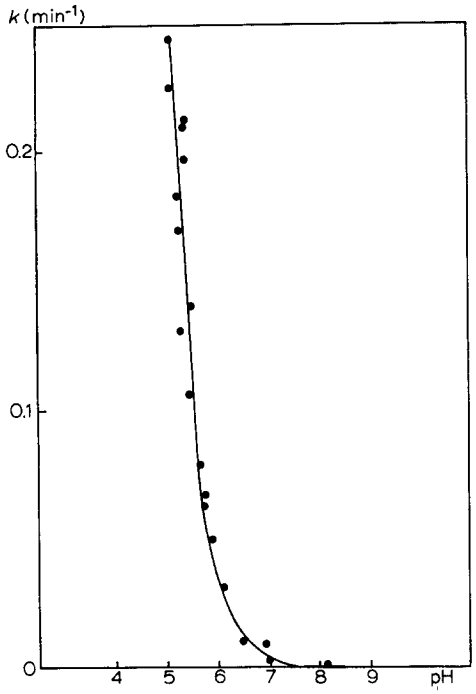

Fig. 5. pH-dependence of the rate of lipase inactivation by EDC and glycine ethyl ester. For the assays, a 2-mg/ml solution of native lipase was reacted at room temperature with 62.5 mM EDC and 1 M unlabeled glycine ester. The pH was adjusted to the desired value and then maintained constant with the aid of a pH-stat.

diimides at pH 7.0. The resulting derivative was inactive. But, activity was fully restored and, consequently, the corresponding bond could be expected to be cleaved, by treatment with hydroxylamine[27]. In the case of lipase, no hydroxylamine-induced reactivation could be observed. Therefore, the reaction of carbodiimides with an hydroxyl group in lipase was considered unlikely.

An additional indication that carbodiimides were largely specific for carboxyl

TABLE II

INFLUENCE OF THE TREATMENT WITH EDC AND GLYCINE METHYL ESTER ON THE LEVEL OF TYROSINE, HISTIDINE AND CYSTEINE IN LIPASE

Inhibition (%)	*Number of residues per mole of lipase*		
	His	*Tyr*	*Cys**
0	9.3	14.7	1.91
27	9.4	14.4	
42	9.2	15.1	
56	9.2	15.3	
66	9.5	15.5	
92	9.5	14.4	1.80

* These residues were determined in the unhydrolyzed protein by the Ellman's technique in presence of 0.3% sodium dodecylsulfate[2].

groups in lipase resulted from the fact that no decrease of the enzyme activity occurred in the absence of added amino acid ester. Only carboxyls are known for generating unstable derivatives with carbodiimides in an aqueous medium[13]. This observation also ruled out the possibility of inactivation being caused by the formation of intramolecular bridges which would not require the participation of amino acid esters.

Catalytic properties of 5N-lipase

Table III shows that treatment of lipase by CMC and norleucine methyl ester induces a parallel drop of activity towards a number of aggregated substrates such as emulsified triolein and tributyrin, and micelles of tripropionin and triacetin. It appears,

TABLE III

LIPASE ACTIVITY TOWARDS VARIOUS AGGREGATED SUBSTRATES AFTER TREATMENT WITH CMC AND NORLEUCINE METHYL ESTER

(a) Purified olive oil emulsion containing bile salts at pH 9.0 and 37 °C. (b) Purified tributyrin emulsion (0.5 ml in 15 ml of 0.1 M NaCl) at pH 8.0 and 25 °C (ref. 4). (c) Solutions of the short-chain glycerides (0.2% for tripropionin; 7% for triacetin) in 0.1 M NaCl at pH 8.0 at 37 °C. These solutions have been shown[18] to contain micelles on which intact lipase is active.

Incubation time (min)	*Remaining activity (%) towards*			
	Emulsions		*Micelles*	
	Triolein (a)	*Tributyrin (b)*	*Tripropionin (c)*	*Triacetin (c)*
30	32	35	36	31
60	20	22	22	21

therefore, that all lipase activities towards this class of substrates is controlled by the same site and that the integrity of this site is affected in some way by the modification of a carboxyl.

By contrast, it was of considerable interest to find that the 5N-lipase derivative, although completely inactive towards the above substrates, still possessed a functional catalytic site that could be titrated by diethyl-*p*-nitrophenyl phosphate. The only difference between the titration of the native and modified form of lipase was that the latter was slightly slower.

Attempted protection of lipase against inactivation by carbodiimides

Protection against carbodiimide inactivation afforded by competitive inhibitors has been successfully employed in recent years for confirming the participation of carboxyls in the active site of trypsin and lysozyme[10,22]. However, very few competitive inhibitors have so far been characterized for lipase, probably because of the uncertainties inherent in the emulsified systems in which lipase activity is usually measured. Among them, the best known at the present time consists of micelles of glycerol 1-octyl ether which have been recently shown in this laboratory to form a high molecular weight complex with lipase and to exert a distinct competitive inhibition effect in the presence of emulsified tributyrin as the substrate (Sari, H., unpublished experiments).

Accordingly, the complex was prepared as described in Material and Methods by

incubation of lipase with a concentrated solution of the ether in 1 M NaCl. The activity of the material migrating unretarded through Sephadex G-200 was reduced 2–3-fold. The reaction with carbodiimides was carried out in 1 M NaCl and in presence of 0.77 mg/ml of α-glycerol octyl ether. But no protection against inactivation could be observed. No protection was also provided by emulsions of oleylalcohol or 1-monocaproin. However, an insolubilization of the modified lipase by the inhibitors and also by the emulsified substrates themselves was noted. This important point will be discussed later in more details.

Finally, the possibility for the diethylphosphoryl group bound to the essential serine to protect adjacent carboxyls against carbodiimides was explored. Fig. 6 shows that 5 carboxyl groups are modified by CMC in diethyl-*p*-nitrophenyl phosphate-inhibited lipase as they are in the native enzyme. The only detectable and somewhat unexpected difference was the faster reaction of one carboxyl, resulting probably from a slight conformational change of the molecules brought about by the binding of the diethylphosphoryl radical.

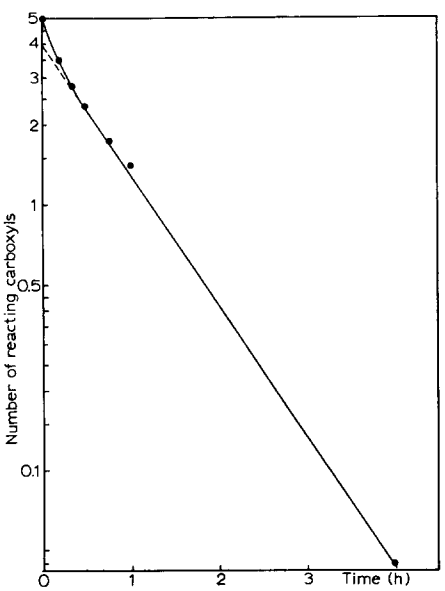

Fig. 6. Time course of the reaction of the diethylphosphoryl derivative of lipase with CMC and norleucine methyl ester. The experimental conditions were the same as for Fig. 3 and Fig. 4 related to the native enzyme. Extrapolation of the linear portion of the curve shows that 4 carboxyls were modified at a rate similar to that observed for the native enzyme (Fig. 4), whereas the fifth group reacted distinctly faster.

Effect of the modification of the carboxyls on lipase stability in 8 M urea

The rate of the denaturation of native and 5N-lipase in 8 M urea was compared by spectrophotometry at 294 nm. This wavelength corresponds to a maximum in the differential spectrum between the native and denatured enzyme. Fig. 7 shows that the denaturation of the 5N-derivative is 14 times faster, suggesting for the modified carboxyls a stabilizing effect on the structure of the enzyme molecule.

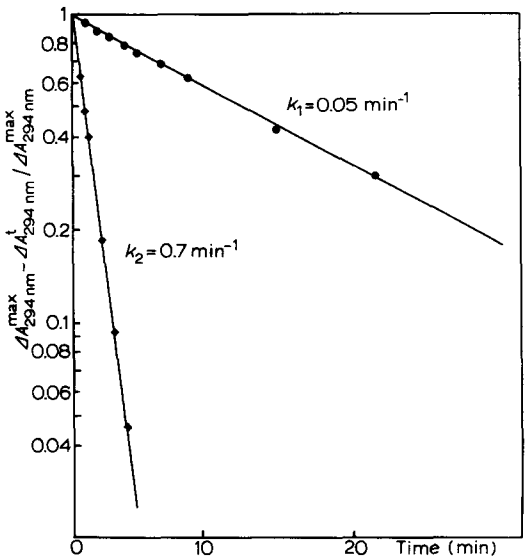

Fig. 7. Denaturation of native (●) and 5N-lipase (■) in 8 M urea at 22 °C. The solutions were buffered at pH 5.6 by 0.2 M sodium acetate.

DISCUSSION

EDC and glycine ethyl ester are considered to be appropriate reagents for the determination of "exposed" (reacting) and "buried" (non-reacting) carboxyls in native proteins[13]. When applied to lysozyme[22] and chymotrypsin[29], respectively, this technique showed that 10 out of 11 and 13 out of 15 groups had reacted. In other words, almost all the carboxyls, as expected from their polar nature, were found to be "exposed" in these enzymes. The few groups observed to be "buried" and consequently abnormal turned out to play an important role in the biological function of the molecules.

The case of lipase appears at first sight to be quite different since the number of reacting carboxyls is reported here not to exceed 14 out of a total of 41. This observation might suggest that a majority of carboxyls in lipase are "buried". Actually, a closer examination of various data indicates that one of the characteristics of lipase in this respect is to contain 14 very reactive carboxyls. The reactivity of these groups is similar and approaches the highest possible value measured in the fully denatured protein. Other carboxyl groups in lipase would undoubtedly have reacted, although at a slower rate, if incubation with EDC had been pursued for a longer period.

Since the 14 carboxyls appeared to display about the same reactivity, their number could not be expected to decrease by using milder conditions or operating during a shorter time. However, utilization of a more bulky carbodiimide and amino acid ester resulted in the obtention of an apparently well-defined derivative of lipase in which 5 carboxyls only were modified. These groups may be assumed to be less sterically hindered than the others.

An interesting feature of the reaction of lipase with carbodiimides was that the modification of the 14 or of the 5 carboxyls led to complete inactivation. Considering that carboxyl modification and inactivation were both pseudo first order and that

rate constants were very close to each other, the correlation method first proposed by Ray and Koshland[31] could be applied. The simplest model in this respect is that lipase contains a single essential carboxyl. But, other models in which several carboxyls are involved are also possible. The exact number of essential carboxyls in lipase will not be known before more specific reagents than carbodiimides are available.

The role played by the carboxyl in lipase activity deserves some comments. The very high reactivity of this group seems to exclude the possibility of it being involved in a charge-relay system similar to that existing in chymotrypsin[5]. Moreover, the observation that the 5N-derivative, although inactive towards the normal substrates of lipase, still possesses a titratable active site and the unsuccessful attempts to protect the carboxyl against carbodiimides by the presence of competitive inhibitors are consistent with the participation of the carboxyl in the structure rather than in the catalytic activity of the enzyme. This assumption is supported by the much faster denaturation of 5N-lipase in 8 M urea when compared to that of native lipase.

A last point of interest would be to know whether the native form of lipase or any other form is stabilized by the carboxyl. It has been postulated for a number of years[31,32] that native lipase is an inactive protein which is activated upon adsorption to an hydrophobic interface. Then, a likely hypothesis would be that the carboxyl is exclusively involved in the stabilization of the functional conformation of lipase resulting from adsorption. This hypothesis receives some experimental support from the observed insolubilization of the modified lipase by the presence of emulsified substrates or inhibitors. It would also explain why the carboxyl is not protected in the native form of the enzyme by the bonds necessary for stabilization.

REFERENCES

1 Desnuelle, P. (1972) in *The Enzymes*, Vol. VII, p. 575, 3rd edn, (Boyer, P. D., ed.), Academic Press, New York
2 Verger, R., Sarda, L. and Desnuelle, P. (1971) *Biochim. Biophys. Acta* 242, 580
3 Maylié, M. F,. Charles, M. and Desnuelle, P. (1972) *Biochim. Biophys. Acta* 276, 162
4 Sémériva, M., Dufour, C. and Desnuelle, P. (1971) *Biochemistry* 10, 2143
5 Blow, D. M., Birktoft, J. J. and Hartley, B. S. (1969) *Nature* 221, 337
6 Shotton, D. M., White, N. J. and Watson, H. C. (1972) *Cold Spring Harbor Symp. Quant. Biol.* 36, 91
7 Kraut, J. (1971) in *The Enzymes*, 3rd edn (Boyer, P. D., ed.) p.547, Academic Press, NewYork
8 Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. and Sarma, V. R. (1967) *Proc. R. Soc. Ser., London B* 167, 378
9 Delamare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D. and Offord, R. E. (1972) *Biochem. J.* 129, 321
10 Eyl, Jr, W. and Inagami, T. (1971) *J. Biol. Chem.* 246, 738
11 Paterson, A. K. and Knowles, J. R. (1972) *Eur. J. Biochem.* 31, 510
12 Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. and Coppola, J. C. (1968) *Brookhaven Symp. Biol.* 21, 24
13 Hoare, D. G. and Koshland, Jr, D. E. (1967) *J. Biol. Chem.* 242, 2447
14 Sémériva, M. and Dufour, C. (1972) *Biochim. Biophys. Acta* 260, 393
15 Benzonana, G. and Desnuelle, P. (1965) *Biochim. Biophys. Acta* 105, 121
16 Verger, R., de Haas, G. H., Sarda, L. and Desnuelle, P. (1969) *Biochim. Biophys. Acta* 188, 272
17 Desnuelle, P., Constantin, M. J. and Baldy, J. (1955) *Bull. Soc. Chim. Biol.* 37, 285
18 Entressangles, B. and Desnuelle, P. (1968) *Biochim. Biophys. Acta* 159, 285
19 Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* 30, 513
20 Guggenheim, E. A. (1926) *Phil. Mag.* 2, 538
21 Carraway, K. L. and Koshland, Jr, D. E. (1972) in *Methods in Enzymology*, Vo. 25, p. 616, (Hirs, C. H. W., ed.), Academic Press, New York
22 Lin, T. S. and Koshland, Jr, D. E. (1969) *J. Biol. Chem.* 244, 505

23 Chang, C. C. and Yang, C. C. (1971) *Biochem. Biophys. Res. Commun.* 43, 429
24 Carraway, K. L. and Koshland, Jr, D. E. (1968) *Biochim. Biophys. Acta* 160, 1972
25 Carraway, K. L. and Triplett, R. (1970) *Biochim. Biophys. Acta* 200, 564
26 Khorana, H. G. (1953) *Chem. Rev.* 53, 145
27 Banks, T., Blossey, B. and Shafer, J. (1969) *J. Biol. Chem.* 244, 6323
28 Riehm, J. P. and Scheraga, H. A. (1966) *Biochemistry* 5, 99
29 Abita, J. P., Maroux, S., Delaage, M. and Lazdunski, M. (1969) *FEBS Lett.* 4, 203
30 Ray, W. J. and Koshland, Jr, D. E. (1961) *J. Biol. Chem.* 236, 1973
31 Sarda, L. and Desnuelle, P. (1958) *Biochim. Biophys. Acta* 30, 513
32 Esposito, S., Séméria, M. and Desnuelle, P. (1973) *Biochim. Biophys. Acta* 302, 293