On the Reaction of Acetic and Maleic Anhydrides with Elastase. Evidence for a Role of the NH_2-Terminal Valine†

Doris Karibian,‡ Charmaine Jones, Arieh Gertler, Keith J. Dorrington, and Theo Hofmann*

ABSTRACT: Porcine pancreatic elastase treated with acetic anhydride in the neutral pH range was reversibly inactivated. At room temperature and pH 7.6 the enzyme was reactivated over a period of about 1 hr. At pH 9.5 reactivation was complete in less than 1 min. All three lysine residues and 6.03 ± 0.5 tyrosine residues were acetylated while the NH_2-terminal valine remained unsubstituted. The group responsible for inactivation has not been identified, but lysine and tyrosine have been ruled out. Hypothetically it is suggested that acetylation of histidine-57 is responsible for the observed effects. At pH 10.5, or at pH 9–9.5 in 2 and 4 M urea, acetic anhydride irreversibly inactivated elastase. There was a direct correlation between the residual activity of partially inactivated elastase and the residual unblocked NH_2-terminal group. Unsubstituted valine-16 was found entirely in the active part of partially inactivated enzyme. Inactive elastase was devoid of a free NH_2-terminal group. Treatment of elastase with maleic anhydride at pH 11.5 caused loss of esterase activity and corresponding loss of free NH_2-terminal valine. Differences in the circular dichroism spectrum between pH 8.5 and 10.6 were compared with differences between pH 4.5 and 2.9 where a conformational change involving the NH_2-terminal valine has previously been demonstrated. The magnitude of the changes and the wavelengths where they occurred were similar in the high and the low pH ranges. Therefore the CD changes occurring between pH 8.5 and 10.6 are indicative of, or at least compatible with, a conformational change in the NH_2-terminal region. The results suggest that the free amino group of valine-16 is required for full enzymatic activity, and in analogy with other serine proteases probably maintains the active conformation by forming an ion pair with aspartic acid-194.

As a result of studies on the chemical modification of porcine pancreatic elastase by nitrous acid (Gertler and Hofmann, 1967) and by 2,4,6-trinitrobenzenesulfonic acid (Rao and Hofmann, 1970) it became desirable to make a comparison between native elastase and elastase modified by acetic anhydride. In a report on the pH dependence of the catalytic activity of elastase Kaplan and Dugas (1969) briefly mention that treatment with acetic anhydride leads to acetylation of the α-amino group of the NH_2-terminal valine and the ϵ-amino groups of all three lysine residues with retention of essentially full activity and suggest that the NH_2-terminal group plays no role in the enzymatic function. While we could readily prepare fully active elastase in which all lysines had been blocked, we were never able to obtain active acetylated elastase in which the NH_2-terminal valine had also reacted. The results of Kaplan and Dugas (1969) are also not readily compatible with earlier studies from this laboratory (Gertler and Hofmann, 1967) or with the X-ray analysis (Shotton and Watson, 1970), which indicate an important role for the valine-16 in maintaining the active conformation of the enzyme. For these reasons the present study of the effect of acetylation on the enzymatic activity was undertaken. Kaplan *et al.* (1971) have published a report on their elegant work on the acetylation of elastase but their work was concerned with the reactivity of the amino groups and not with effects of acetylation on enzymatic activity.

Experimental Section

Materials. Porcine pancreatic elastase was prepared as described (Gertler and Hofmann, 1970). $AcAla_3OMe$[1] was from Cyclo Chemical Co. (Los Angeles, Calif.) and CGN from Sigma Chemical Co. Other chemicals used were of the highest purity available.

Methods. Enzymatic Activities. Esterase activities with $AcAla_3OMe$ as substrate were determined in a pH-Stat as described (Gertler and Hofmann, 1970) except that mM substrate concentrations were used with 0.02 M NaOH as titrant.

Activities with CGN as substrate were determined spectrophotometrically as described (Rao and Hofmann, 1970) with a Unicam SP 800 spectrophotometer. An external recorder was used for scale expansion.

Elastolytic activities were estimated by a direct spectrophotometric method (Ardelt *et al.*, 1970) with small modifications (Gertler and Hofmann, 1970).

NH_2-Terminal Groups. These were determined by the method of Stark and Smyth (1963) with the modifications described previously (Gertler and Hofmann, 1967).

Free ϵ-Amino Groups. These were estimated from the homocitrulline content of samples used for NH_2-terminal analysis by the Stark and Smyth method (1963). In some experiments a DNFB method described previously (Gertler, 1971) was used.

Acetyl Groups on Tyrosine Residues. The number of labile acetyl residues was determined spectrophotometrically by measuring the absorbance changes at 244 nm when the acetylated protein was treated at pH 11.5 by the method of Simpson *et al.* (1963), as described by Karibian *et al.* (1968). The acetylation was also estimated from difference spectra of the acetylated

† From the Departments of Biochemistry, University of Toronto, Toronto, Canada, M5S 1A8, and Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel (A. G.). *Received December 28, 1973.* This work was supported by research grants from the Medical Research Council of Canada (MT-1982 and MT-4259).

‡ Present address: CNRS Laboratoire de Chimie Bacterienne, 13274 Marseilles, France.

[1] Abbreviations used are: DNFB, 2,4-dinitro-1-fluorobenzene; $AcAla_3OMe$, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; CGN, benzyloxycarbonylglycine *p*-nitrophenyl ester.

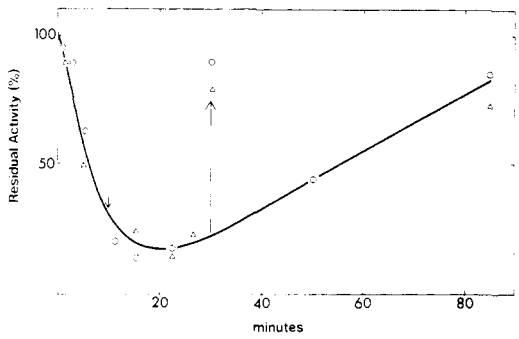


FIGURE 1: Reversible inactivation of elastase by acetic anhydride at pH 7.6, 34°. Elastase, 0.35 mg/ml (14 μM) in 2 ml of 0.02 M phosphate in pH-Stat cell. Ten lots of 0.2 μl of acetic anhydride were added at 1-min intervals (↓ indicates last addition). The pH was maintained at 7.6 by titration with 2 N NaOH. At intervals samples were taken for assays: 25 μl for assay with $AcAla_3OMe$ (○) in another pH-Stat; 50 μl for spectrophotometric assay with CGN (△). ↑ at 30 min indicates sample reactivated by raising the pH to 9.5 for 2 min before assay.

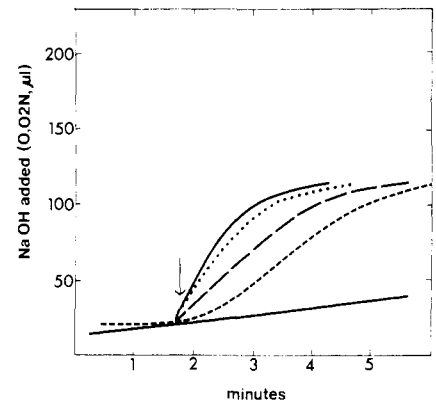


FIGURE 2: Recorder tracings from pH-Stat assay of elastase with $AcAla_3OMe$. Conditions: 10^{-3} M substrate, 8 μg of elastase in 2 ml of 0.015 M Tris–0.045 M KCl (pH 8.0), 30°; ↓ point of enzyme addition; (—) before acetylation; (- - - -) after acetylation for 9 min as described in Figure 1; (— —) after acetylation for 10 min followed by 40-min incubation; (. . . . .) after reactivation for 2 min at pH 9.5; sloping line, approximate initial rate for sample after acetylation for 9 min.

protein before and after treatment with 1 M NH_2OH for 1 hr at pH 7.5 and 25° (Karibian *et al.*, 1968).

Acetylation of Elastase. All acetylation experiments were carried out in thermostated vessels with a Radiometer pH-Stat at a variety of temperatures. Anhydrous acetic anhydride was slowly added to elastase solutions (14–20 μM in various buffers) from a micrometer syringe to give concentration changes of the reagent of 0.5–10 mM/min over periods of 10–30 min. The total amount of acetic anhydride added corresponded to 1000- to 2000-fold molar excesses. The pH was kept constant by automatic titration using another micrometer syringe with 0.5–2 N NaOH as titrant depending on the reaction volumes. These varied from 1.5 to 10 ml, the larger volumes being required for NH_2-terminal analyses.

Maleylation of elastase was also carried out in a Radiometer pH-Stat at 2°. Elastase solutions in 0.01 M sodium borate buffer (pH 8.9) (2.5 ml, 10 mg/ml) were adjusted to the final pH by adding NaOH. The maleylation at pH 11.5 was carried out by addition of 5-μl portions of 4 M maleic acid anhydride in dioxane to give 200- to 1200-fold molar excesses. The reaction was allowed to proceed for 25 min. The pH was kept constant within the range 11.3–11.7 by titration with 2.5 N NaOH. In the control experiment dioxane without maleic anhydride was added. The maleylation at pH 8.9 was performed in a similar way but maleic anhydride was 1 M and added in 50-μl portions, the reaction time was 10 min, and the titrant used was 1 N NaOH. After the reaction was complete the pH was lowered to 8.7 with 1 N HCl and the reaction mixture was exhaustively dialyzed against 0.01 M sodium borate buffer at pH 8.7 and 4°. The concentration of elastase was then estimated by measuring the extinction at 280 nm assuming the specific extinction $E_{1\,cm}^{1\%} = 20.2$ (Shotton, 1971); a correction was made for the content of maleyl-amino groups (Butler *et al.*, 1969).

The dialyzed material was used for the estimation of esterolytic and elastolytic activities and for determination of α- and ϵ-amino groups.

Circular Dichroism (CD). CD spectra were recorded on a Durrum-Jasco spectropolarimeter equipped with the SS-20 CD modification as described (Dorrington and Hofmann, 1973). The results are presented as mean residue ellipticity (deg cm^2 $dmol^{-1}$). A mean residue weight of 108 was calculated from the known composition (Shotton, 1971).

Results

Effect of Acetic Anhydride on Elastase. Reversible Inactivation. Activities. Initial experiments with acetic anhydride in the neutral pH range indicated that elastase was readily inactivated by acetic anhydride. However, reproducible results were difficult to obtain until it was discovered that the inactivation was reversible. This phenomenon was therefore first investigated. Figure 1 gives the result of an acetylation experiment carried out at pH 7.6 and 34°. There was a rapid drop of $AcAla_3OMe$ and CGN esterase activities during the addition of the reagent. A minimum of around 15% of the original activity was reached and this was followed by a slow recovery which reached 72–85% of the original activity in 75 min after the addition was complete. On standing overnight the activity returned to 85–100%. The same reactivation could be obtained any time after the reagent had been added by bringing the solution to pH 9.5 for 1–2 min and returning it to neutral (as shown for example by ↑ in Figure 1).

The reactivation could also be seen during the course of the esteratic assay with $AcAla_3OMe$ at pH 8.0, 30° (Figure 2). Normally the assay is linear under the conditions used until about 50% of the substrate is hydrolyzed. However, when samples of the acetylation mixture with low activity were assayed the time curves became markedly sigmoidal suggesting that inactivated enzyme became reactivated during the assay. Because of the sigmoidal assay curves the minimum activities given in Table I and Figure 1 were difficult to estimate and are subject to considerable error. They were obtained from slopes of the lower portions of the sigmoidal curves (shown by the sloping straight line in Figure 2). The spectrophotometric assays with CGN showed the same phenomenon. The reversible inactivation was studied in several experiments under different conditions (Table I). The lowest values for residual activities ranged between 6 and 30% of the original; after incubation at pH 9.5 the activities returned to 85–110%.

In the experiments with maleic anhydride described previously (Gertler, 1971) and below, a similar reversible loss of esterolytic activity was observed; this was not further investigated.

Acetylation of Amino Acids. The most probable explanation for the reversible loss of activity is that an amino acid side chain involved in the active site becomes acetylated with concurrent loss of activity which is regained when the acetyl group is spontaneously hydrolyzed. An attempt was made to identify the residue involved. Table I shows that in the three experiments where the NH_2-terminal valine-16 had been determined it was freely available to the cyanate reagent of Stark and

TABLE I: Conditions under Which Acetic Anhydride Caused No Irreversible Inactivation.[a]

pH	Solvent	Temp, °C	Acetic Anhydride Addition: Total (mM)	Acetic Anhydride Addition: Rate (mM/min)	Activities (in % of initial activity): $AcAla_3OMe$ Minimal	$AcAla_3OMe$ After Recovery[b]	CGN Minimal	CGN After Recovery[b]	NH_2-Terminal Valine (mol/mol)	Homocitrulline (mol/mol)
5.2	5 mM acetate	25	50	5	-[c]	-	-	90	-	-
6.7	5 mM $NaHCO_3$	25	50	5	-	-	20	85	-	-
	2 M urea	4	50	5	-	-	10	110	-	-
	2 M urea	25	50	5	-	-	12	95	0.92	<0.05
7.6	20 mM phosphate	25	32	2	30	86	-	-	0.85	<0.05
	15 mM SDS[d]	25	50	5	-	-	25	100	-	-
	20 mM phosphate	35	10	1	15	85	7	86	-	-
	20 mM phosphate–0.1 M KCl	30	10	1	32	110	25	110	0.9	<0.05
	20 mM phosphate–0.1 M KCl	35	10	1	6	86	16	85		

[a] Elastase concentrations were between 14 and 25 μM in all experiments. [b] After recovery means activity found 1–2 hr after the end of acetic anhydride addition or after treatment for 1–2 min at pH 9.0. [c] - indicates not determined. [d] SDS, sodium dodecyl sulfate.

Smyth (1963) under the conditions used by Gertler and Hofmann (1967) while all three lysines had been acetylated as shown by the absence of homocitrulline. In the control samples 2.2–2.5 mol of homocitrulline/mol of elastase was obtained. This corresponds to a recovery of 73–85% if it is assumed that all lysine residues had been converted to homocitrulline by cyanate. This recovery agrees well with that of 70–83% given by Stark and Smyth (1963).

Tyrosine residues also become acetylated under the conditions shown in Table I. In ten experiments a value of 6.03 ± 0.5 residues was obtained as determined by the method of Karibian *et al.* (1968) from the change in absorbance at 244 nm. Table II gives the results of an experiment in which the acetyltyrosine content of a sample (II) taken at minimal activity (6%) was compared with that of a sample (III) taken after reactivation. No significant difference was found between the two samples.

NH_2-terminal valine was also determined on sample II in order to try to eliminate the possibility—albeit unlikely—that acetylation of the α-amino group had occurred, but that the acetyl group was subsequently removed through internal or external enzymatic catalysis. The treatment of sample II, namely precipitation in an acidified organic solvent at low temperature (see footnote *b*, Table II) followed by denaturation in SDS at pH 3 (Gertler and Hofmann, 1967), was designed to prevent the loss of the acetyl group.

Irreversible Inactivation. The experiments described above show that under a variety of conditions in the neutral pH range, treatment with acetic anhydride causes reversible but not irreversible inactivation and does not lead to acetylation of NH_2-terminal valine.

In subsequent experiments acetylations were carried out at 35° and pH 10.5 and also in urea at pH 9–10 at 4 and 22°. Esterase activities were measured and NH_2-terminal analyses carried out. Some of the experiments are described in Table III. They show a clear correlation between the residual esterase activity and the free amino group of valine-16. The results of the experiments in Table III and a number of others, as well as the results of maleylation described below, are presented graphically in Figure 3. The close correlation between residual activity and residual free NH_2-terminal groups is evident.

Further evidence for this correlation was obtained in experiments in which active and inactive elastase was separated. It was noticed during the experiments leading to irreversible inac-

TABLE II: Effect of Acetic Anhydride on Amino Acids.[a]

Time (min)	Sample	Activity on $AcAla_3OMe$ (% of I)	NH_2-Terminal[b] Valine (mol/mol)	Homocitrulline (mol/mol)	Acetyltyrosine[c] (ΔOD at 244 nm)	Acetyltyrosine[c] (mol/mol)
0	I before acetylation	100	0.95	2.4	0	0
10	II before reactivation	6	0.85	0.05	0.480	5.8
30	III after reactivation	86	0.91	0.05	0.520	6.3

[a] Elastase (10 ml, 14 μM) in 0.02 M phosphate at 7.6, 34°, was treated with 2 μl of acetic anhydride added over 10 min. For reactivation the pH was raised to 9.5 for 2 min. Samples (25 μl) were taken for assay at intervals. [b] Samples (2.5 ml) were removed for NH_2-terminal assay. They were added to 25 ml of acidified acetone–ethanol–ether (10:10:1, by vol) at −25°. The precipitate was treated as described (Gertler and Hofmann, 1967). [c] Samples (0.5 ml) were analyzed by measuring the difference spectra before and after treatment with 1 M hydroxylamine (Karibian *et al.*, 1968).

TABLE III: Irreversible Inactivation of Elastase by Acetic Anhydride.[a]

pH	Solvent	Temp, °C	Activities (% of initial)		Amino Acids (mol/mol)	
			$AcAla_3OMe$	CGN	NH_2-Terminal Valine	Homocitrulline
10.5	0.05 M borate	35	20	-[b]	0.18	0.05
9.0	2 M urea	4	-	65	0.53	0.05
9.5	2 M urea	4	-	40	0.46	0.05
9.0	4 M urea	4	30	-	0.29	0.05

[a] Elastase concentrations were between 10 and 100 μM; acetic anhydride was added at a rate of 6 mM/min to a final amount of 100 mM. [b] - indicates not determined.

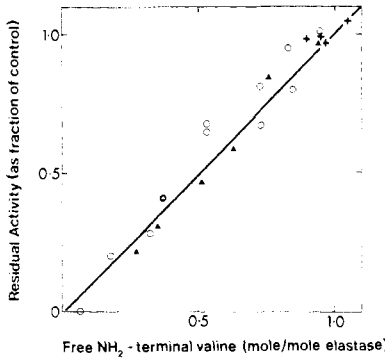
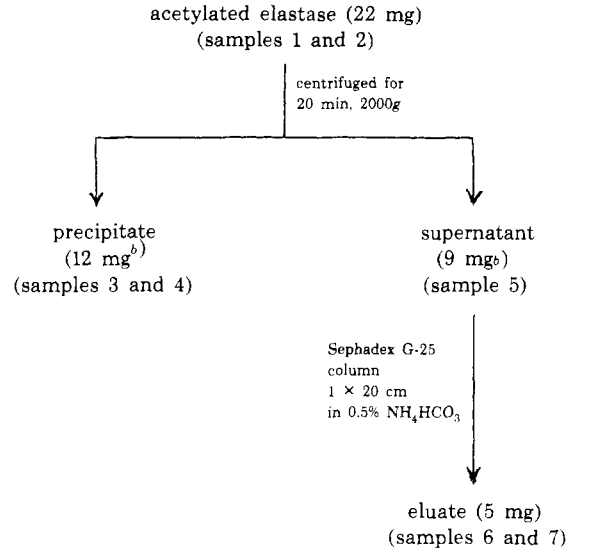


FIGURE 3: Correlation between acylation of NH_2-terminal valine and irreversible loss of esterase activity: (○) acetylated with acetic anhydride at pH 7–10.5 with or without urea, from many different experiments; (▲) maleylated at pH 11.5 as described under Methods; (+) controls.

tivation that protein precipitates formed in the reaction vessels. Scheme I shows an outline of an experiment in which the precipitate was separated from the soluble protein. The soluble protein was further purified on a Sephadex G-25 column to remove urea. The protein was analyzed at the different stages of separation for specific activity, for free NH_2-terminal valine, for homocitrulline, and for acetylated tyrosine (soluble fractions only). The results (Table IV) show that the insoluble protein was inactive and devoid of free NH_2-terminal valine (sample 3), while the soluble enzyme was fully active after chromatography and had retained a fully reactive NH_2-terminal (sample 6). The increase in specific activity during chromatography can be accounted for by assuming that some inactive protein had become insoluble and was retained in the column.

SCHEME I: Separation of Soluble and Insoluble Elastase after Acetylation.[a]



[a] Elastase (1 mg/ml, 10 ml) in 2 M urea (pH 9.5), 25°, was acetylated with 1.32 mmol of acetic anhydride added over a period of 10 min. [b] Recovery figures are corrected for samples removed at each stage of the separation.

At each stage (except the "supernatant," Scheme I) a control from which the cyanate reagent had been omitted was carried through the Stark and Smyth procedure (1963). These controls were necessary because the Stark and Smyth procedure (1963) is an indirect analysis and measures as "NH_2-terminal" amino acid the hydrolysis product of the corresponding

TABLE IV: Analysis of Fractions from Partially Inactivated Acetylated Elastase.[a]

Samples	Specific Activity with CGN (as % of untreated)	Valine Found[b] (mol/mol)	Homocitrulline (mol/mol)	Tyrosine (mol/mol)
1 Acetylated elastase	29	0.32	0.03	*d*
2 Acetylated elastase control[c]		0.02		
3 Precipitate	0	0.08	0.04	*d*
4 Precipitate control[c]		0.05		
5 Supernatant	67	0.73	0.04	6.7
6 Eluate	91	0.93	0.05	5.8
7 Eluate control[c]		0.01		

[a] Fractions prepared according to Scheme I. [b] This is valine isolated as hydantoin and subsequently hydrolyzed. [c] Controls were treated as the test samples, but KCNO was omitted (see also text). [d] Could not be determined, because of precipitate.

TABLE V: Effect of Maleylation of Elastase on Esterolytic and Elastolytic Activities and on the Content of NH_2-Terminal Valine and ε-Amino Groups of Lysine.[a]

pH	Total mmol of Maleic Anhydride Added	Relative Enzymatic Activity on $AcAla_3$-OMe	Elastin	Amino Acids (mol/mol) NH_2-Terminal Valine	Free Lysine[b]
Untreated elastase		1.00	1.00	1.05	3.10
8.9	0.4	0.97	0.03	0.94	0.12
8.9	0.0	0.99	1.03	0.95	2.95
11.5	1.2	0.22	0.03	0.27	0.03
11.5	0.9	0.31	0.05	0.35	0.22
11.5	0.6	0.47	0.04	0.51	0.02
11.5	0.4	0.59	0.02	0.63	0.20
11.5	0.2	0.85	0.03	0.76	0.10
11.5	0.0	0.98	1.01	0.97	2.84

[a] For experimental details see Methods. [b] The amount of the "free lysine" was calculated by measuring the lysine obtained after acid hydrolysis of elastase which was treated with DNFB after maleylation. The values obtained were subtracted from that of untreated elastase to give "free lysine."

hydantoin. The procedure for isolation of the hydantoin is nonspecific and will separate any amino acid or peptide derivative without a positive charge. However, acetylvaline is not isolated during this procedure because during the cyclization of the carbamyl-protein acetyl groups are hydrolyzed. This is shown by sample 4. Furthermore it was possible that during acetylation at pH 9.5 in urea sufficient cyanate could have formed to block the NH_2-terminal and prevent its acetylation. As Table IV (samples 2, 4, and 7) shows these controls showed at the most traces of valine. The valine recovered in the test samples 1, 3, 5, and 6 must therefore have originated solely from valine which had a free amino group at the end of the acetylation step.

The absence of homocitrulline showed that all lysine residues had been acetylated. Unfortunately it was not possible to estimate the number of acetylated tyrosines in samples with precipitate. In samples 6 and 7 about six tyrosines had been acetylated. This is the same as that found in the acetylations at lower pH (Table II).

Effect of Maleic Anhydride on Elastase. Maleic anhydride has been used previously to study the interaction of elastase with elastin (Gertler, 1971) During the course of these studies the effect of maleylation on the esterase activity and on the NH_2-terminal was also followed. Some of the results are presented in Table V. As shown previously (Gertler, 1971) maleylation at pH 8.9 had no effect on the esterase activity and on the NH_2-terminal valine, but led to almost complete loss of elastolytic activity. When the pH was raised to 11.5 and maleic anhydride added in increasing amounts a progressive loss of the NH_2-terminal valine occurred which was accompanied by a loss of esteratic activity. The correlation between these parameters is shown in Figure 3. The reactivity of the NH_2-terminal was low, since with the smallest amount of maleic anhydride added (200-fold molar excess over elastase) only about 20% of valine-16 had reacted, while the three lysine residues appeared to be completely substituted. Increasing amounts of maleic anhydride led to increasing substitution of the NH_2-terminal group. Unfortunately maleyl elastase is unstable below pH 6.0 and denatures irreversibly. Therefore it was not possible to remove the maleyl groups and regenerate enzymatic activity (see also Gertler, 1971).

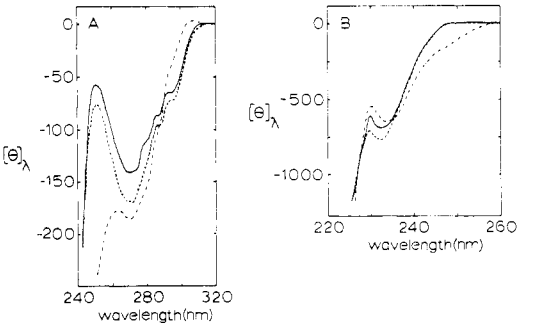


FIGURE 4: pH dependence of circular dichroism spectra of elastase. (A) Between 320 and 250 nm at pH 2.95 (—), 4.2 (- - - - -), 8.5 (. . . . .), and 10.6 (-·-·-). Protein concentration 0.65 mg/ml. (B) Between 260 and 230 nm at pH 2.9 (—), 4.5–8.5 (- - - - -), and 10.6 (-·-·-). Protein concentration 0.15 mg/ml. The units of mean residue ellipticity, $[\theta]$, are deg cm^2 $dmol^{-1}$.

Circular Dichroism Spectrum. Although the effect of pH on the CD spectrum of elastase has been reported by Gorbunoff and Timasheff (1972) the specific purpose of our studies was to compare CD changes due to the N ⇄ R transition between pH 4.2 and 2.9 (Wasi and Hofmann, 1968) with the changes in the alkaline pH region. The spectra, between 250 and 320 nm, for pH values at 2.95, 4.2, 8.5, and 10.6 are shown in Figure 4A. The spectrum at pH 8.5 is similar to that at pH 4.2 except that the 251-nm band has decreased in magnitude. At pH 2.95 the 270-nm band decreases and the 251-nm band increases in magnitude relative to pH 4.2. At pH 10.6 the 251-nm band, seen at pH 8.5, shows an apparent shift to 263 nm and a decrease in magnitude. These data show that the well-defined N ⇄ R transition produces less pronounced CD changes between 250 and 270 nm than the transfer of the protein to alkaline conditions.

The spectra between 225 and 260 nm are shown in Figure 4B. At pH 4.5 and 8.5 they are identical. The spectrum taken at pH 2.9 shows a small decrease in the 233-nm band and an increase in the 229-nm transition. At pH 10.6 the 229-nm band is increased in magnitude and the negative band decreases and shifts to 234 nm. In this case the changes due to the N ⇄ R transition are qualitatively similar but quantitively less marked than those accompanying the pH change from 8.5 to 10.6.

Below 230 nm the spectra at pH 2.9 and 4.5 were the same as those obtained by Gorbunoff and Timasheff (1972) between pH 5 and 11. This spectral region is surprisingly insensitive to the pH-dependent conformational changes.

Discussion

The results described in the preceding section show that acetylation had two separate effects on the esterase activity of elastase. In the pH range from 5.2 to 7.6 at different temperatures and with a variety of additions loss of esterase activity was observed which was spontaneously but slowly reversible. Attempts were made to identify the group whose acetylation caused this reversible inactivation. The amino groups of the three lysine residues could be ruled out because they remained fully acetylated after complete recovery of activity. In contrast no evidence for acetylation of valine-16 could be found. The failure of valine-16 to react with acetic anhydride in the neutral pH range is entirely compatible with the very low reactivity of this residue noted by Kaplan *et al.* (1971). Reversible acetylation of the NH_2-terminal is unlikely because of the very mild conditions used to denature elastase before determining

the NH_2-terminal. Ghelis *et al.* (1970) were able to deacetylate isoleucine-16 in δ-chymotrypsin and obtain reactivation. However, treatment with 4 M hydroxylamine was required and reactivation was slow and only partial, and followed by general denaturation. No significant difference was found either between the number of tyrosines acetylated (about 6) in the inactivated and the reactivated form. (This number, incidentally, is similar to the number of tyrosines acetylated by acetylimidazole (Gorbunoff and Timasheff, 1972).)

A possible site of transient acetylation is the active site serine (serine-195) which is presumed to be acylated during elastase-catalyzed hydrolysis of *p*-nitrophenyl esters (Bender and Marshall, 1968). Also in experiments with trypsin (Houston and Walsh, 1970) serine-195 (or serine-183 in their numbering system) could be acetylated by *N*-acetylimidazole. However, deacetylation of these residues at pH 7.6 is fast and should be compatible with the rate of hydrolysis of *p*-nitrophenyl acetate. Under the assumption that the deacetylation step for elastase-catalyzed *p*-nitrophenyl acetate hydrolysis is rate limiting, the half-life of the acetyl-enzyme at pH 7.43 and 25° can be calculated from k_{cat} (Bender *et al.*, 1966) and comes to about 35 sec. The half-time of reactivation of acetylated elastase in these experiments, however, is of the order of 35 min (Figure 1) at pH 7.6 and 34°. Hence the inactivation is not due to acetylation of serine-195 either.

Another possibility is histidine-57. This residue is known from the X-ray analysis (Shotton and Watson, 1970) to be in close profimity to serine-195 and is presumed to play a crucial role in the catalytic function of the enzyme (Shotton and Watson, 1970). The transient inactivation of elastase by acetylation affects equally the esterase activities with a specific substrate, $AcAla_3OMe$, and a nonspecific substrate, CGN. This suggests a direct effect on a catalytically essential residue. Acetylation of the imidazole ring can be expected to interfere with the function of the active site serine. The ready deacetylation is compatible with the known lability of *N*-acetyl compounds. It is of interest to note that Houston and Walsh (1970) during acetylation of trypsin by *N*-acetylimidazole observed inactivation by acetylation of a group which at pH 7.6 underwent slow spontaneous deacetylation with regeneration of activity. The nature of this effect and the time scale of reactivation were similar to those observed in our experiments (Figure 1). Houston and Walsh (1970) were unable to identify the group but suggested on the basis of circumstantial evidence and on structural grounds that histidine-46 (the equivalent of histidine-57) was the group which had undergone reversible acetylation. Because of the lability of this acetyl group isolation of the acetyl peptide which would give evidence for the attachment site has not been possible.

As indicated in the introduction the present studies were carried out in order to contribute to our understanding of the possible role of the NH_2-terminal amino group of valine-16 in the catalytic function of the enzyme. As the X-ray analysis has shown this group forms a deeply buried ion pair with aspartic acid-194 in the interior of the molecule (Shotton and Watson, 1970). Since the zymogen proelastase is devoid of a NH_2-terminal valine (Gertler and Birk, 1970) it is reasonable to assume that the activation proceeds through cleavage of a peptide involving valine-16 and that the formation of the ion pair is part of the activation process. In chymotrypsin a considerable volume of evidence is available which shows the essential[2] role of t e ion pair in maintaining the *fully active* conformation of the enzyme (Hess, 1971) although some recent experiments (Agarwal *et al.*, 1971; Blair *et al.*, 1971) are somewhat difficult to reconcile with this concept. Trypsin also has an analogous ion pair (Stroud *et al.*, 1971) and evidence has been presented that modification of the NH_2-terminal isoleucine leads to loss of activity (Scrimger and Hofmann, 1967; Robinson *et al.*, 1973). Thrombin, too, has an equivalent NH_2-terminal isoleucine which appears to be required for enzymic activity (Magnusson and Hofmann, 1970). The amino acid sequence of the first four residues of the homologous serine proteinases have undergone only small conservative mutational changes. Taken together all these considerations suggest an important functional role for the NH_2-terminal amino group and the ion pair in the related homologous serine proteinases.

The acetylation and maleylation experiments leading to irreversible inactivation show the close correlation between substitution of the terminal amino group and loss of activity and are strongly suggestive of a functional role. Under a variety of conditions described above—and others not reported—we have been unable to obtain modification of the NH_2-terminal without loss of activity. This was also true for the modification experiments reported previously (Gertler and Hofmann, 1967; Rao and Hofmann, 1970).

Three major points have been raised against an essential functional role.

(a) Kaplan and Dugas (1969) mention that they were able to acetylate valine-16 at pH 7.5 and retain an active enzyme. The experiments reported in this paper are not compatible with this. Kaplan and Dugas based their finding on the fact that they were unable to detect a free NH_2-terminal valine by the cyanate method of Stark and Smyth (1963) or by the 2,4-dinitrofluorobenzene method. During our earlier studies (Gertler and Hofmann, 1967) we sometimes failed to detect free NH_2-terminal valine in elastase by the Stark and Smyth method until we modified it and included an extensive denaturation by sodium dodecyl sulfate at pH 3. It appears that without this step the NH_2-terminal can remain inaccessible to the cyanate reagent.

(b) There is an apparent discrepancy between the $pK_{(app)}$ = 9.7 of valine-16 measured by acetylation by the competitive labeling procedure of Kaplan *et al.* (1971) and the $pK_{(app)}$ values obtained from kinetic experiments (pK = 10.5–11, Gertler and Hofmann, 1970; pK = 11.4, Geneste and Bender, 1969). However, this difference in apparent pK can be explained if one assumes that the role of valine-16 is one of involvement in a conformational equilibrium which controls the structure of the active site. If the equilibrium constant for such a conformational transition depends on the presence or absence of substrate and possibly also on the structure of the latter then considerable differences between the apparent pK values measured by acetylation and those measured kinetically can be expected.

(c) In chymotrypsin a large pH-dependent change in the circular dichroism has been associated with a conformational

[2] In the following discussion the term "essential" for the functional role of the NH_2-terminal amino group of serine proteinases which is liberated during activation is used in the sense that the group is essential for the *optimal* catalytic efficiency. It should be kept in mind, however, that trypsinogen, the so-called inactive zymogen, has low intrinsic activity (Kay and Kassel, 1971) and reacts slowly with the active-site titrant *p*-nitrophenyl-*p*-guanidinobenzoic acid (Morgan *et al.*, 1972). Also, the active site serine of both trypsinogen and chymotrypsinogen reacts slowly with diisopropyl fluorophosphonate (Morgan *et al.*, 1972) indicating that the active site is in a partly functional state with a low catalytic efficiency. It remains to be investigated whether "inactive" forms of elastase, such as proelastase and chemically modified enzyme, retain a functional active site and low levels of activity. Unfortunately it was not possible to examine the irreversibly inactivated elastase, which had a blocked NH_2-terminal group, for reactivity with active-site titrants or pseudosubstrates because it was insoluble and appeared to be completely denatured.

change due to the deprotonation of isoleucine-16 and consequent rupture of the ion pair with aspartic acid-194 (see Hess, 1971). It was concluded that the ion pair controls the fully active conformation. The absence in elastase of a comparably large CD change in the alkaline pH range has been interpreted as showing the absence of a conformational change due to deprotonation of valine-16. The CD experiments reported in this paper show that this conclusion is not warranted. Our studies confirm the findings of Gorbunoff and Timasheff (1972) that only small CD changes occur between pH 8.5 and 10.6. However, these changes are similar in magnitude and occur in the same wavelength range as the CD changes due to the N $\rightleftarrows$ R transition. This transition which occurs between pH 4.2 and 2.9 is characterized by a large change in the binding of 8-anilino-1-naphthalenesulfonic acid (Wasi and Hofmann, 1968) and by an increase in the reactivity of the NH_2-terminal valine toward nitrous acid (Gertler and Hofmann, 1967). Originally a 20-fold increase was reported, but subsequent detailed kinetic studies of model compounds and various proteins showed that because of intrinsic pH dependence (T. Hofmann, unpublished) and ionic strength effects (Kurosky and Hofmann, 1972) correction factors were necessary. The corrected rate constants for the nitrosation of the NH_2-terminal, k''' (as defined by Kurosky and Hofmann, 1972), are: N form of elastase (at pH 4.2) $k''' = 0.2$ min^{-1} M^{-2} and R form (at pH 3.0) $k''' = 32$ min^{-1} M^{-2}. The latter is similar to that of α-amino groups in model compounds and unfolded proteins ($k''' = 30$–50 min^{-1} M^{-2}). The N $\rightleftarrows$ R transition therefore brings about a 160-fold increase in the reactivity of valine-16 and leads to its full exposure.

The CD changes observed between pH 8.5 and 10.6 therefore do not rule out a localized conformational change involving rupture of the ion pair between valine-16 and aspartic acid-194 and at least partial exposure of valine-16.

The acylation experiments reported here show that valine-16 becomes accessible to the reagents above pH 9.0 or in urea at pH 9.0–9.5. No kinetic studies were undertaken since Kaplan *et al.* (1971) have published an extensive study of acetylation kinetics of the primary amino groups in elastase. They showed that up to approximately pH 10.5 valine-16 has a reduced reactivity toward acetic anhydride. Thus it appears that the NH_2-terminal does not become fully exposed.

In summary, it can be said that while the experiments presented here do not provide conclusive evidence for an essential role (as defined in footnote 2) of the NH_2-terminal, they nevertheless strongly suggest such a role and are not readily compatible with a mechanism in which it plays no role. On this basis it is reasonable to infer that the ion pair between valine-16 and aspartic acid-194 is required for maintaining the fully active conformation. The ion pair thus assumes a similar role to the isoleucine-16–aspartic acid-194 ion pair of trypsin and chymotrypsin (Robinson *et al.*, 1973). It remains an open question, however, whether the major driving force for the transition to an inactive state at high pH is the rupture of the ion pair bond or another interaction, such as one controlled by a tyrosine as suggested by Rao and Hofmann (1970) and Kaplan *et al.* (1971) or the disruption of the protein by general nonspecific electrostatic repulsion as proposed by Gorbunoff and Timasheff (1972). In any case the ion pair would make an important contribution to the structural stability of the active site.

Acknowledgments

The authors are grateful for many fruitful discussions with Dr. H. Kaplan. They would like to thank Mr. D. Duthie and Mr. C. Yu for the amino acid analyses.

References

Agarwal, S. P., Martin, S. J., Blair, T. T., and Marini, M. A. (1971), *Biochem. Biophys. Res. Commun. 43,* 510–515.

Ardelt, W., Ksiezny, S., and Niedzuriedzka-Namyslowska, I. (1970), *Anal. Biochem. 34,* 180–187.

Bender, M. L., Begué-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, D. R., Kézdy, F. J., Killheffer, Jr., J. V., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Amer. Chem. Soc. 88,* 5890–5913.

Bender, M. L., and Marshall, T. H. (1968), *J. Amer. Chem. Soc. 90,* 201–207.

Blair, T. T., Marini, M. A., Agarwal, S. P., and Martin, C. J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 14,* 86–88.

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112,* 679–689.

Dorrington, K. J., and Hofmann, T. (1973), *Can. J. Biochem. 51,* 1059–1065.

Geneste, P., and Bender, M. L. (1969), *Proc. Nat. Acad. Sci. U. S. 64,* 683–685.

Gertler, A. (1971), *Eur. J. Biochem. 23,* 36–40.

Gertler, A., and Birk, Y. (1970), *Eur. J. Biochem. 12,* 170–176.

Gertler, A., and Hofmann, T. (1967), *J. Biol. Chem. 242,* 2522–2527.

Gertler, A., and Hofmann, T. (1970), *Can. J. Biochem. 48,* 384–386.

Ghélis, C., Garel, J. R., and Labouesse, J. (1970), *Biochemistry 9,* 3902–3913.

Gorbunoff, M. J., and Timasheff, S. N. (1972), *Arch. Biochem. Biophys. 152,* 413–422.

Hess, G. P. (1971), *Enzymes, 3rd Ed. 3,* 239–243.

Houston, L. L., and Walsh, K. A. (1970), *Biochemistry 9,* 156–166.

Kaplan, H., and Dugas, H. (1969), *Biochem. Biophys. Res. Commun. 34,* 681–685.

Kaplan, H., Stevenson, K. J., and Hartley, B. S. (1971), *Biochem. J. 124,* 289–299.

Karibian, D., Laurent, C., Labouesse, J., and Labouesse, B. (1968), *Eur. J. Biochem. 5,* 250–269.

Kay, J., and Kassel, B. (1971), *J. Biol. Chem. 246,* 6661–6665.

Kurosky, A., and Hofmann, T. (1972), *Can. J. Biochem. 50,* 1282–1295.

Magnusson, S., and Hofmann, T. (1970), *Can. J. Biochem. 48,* 432–437.

Morgan, P. H., Robinson, N. C., Walsh, K. A., and Neurath, H. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3312–3316.

Rao, L., and Hofmann, T. (1970), *Can. J. Biochem. 48,* 1249–1259.

Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 420–426.

Scrimger, S. T., and Hofmann, T. (1967), *J. Biol. Chem. 242,* 2528–2533.

Shotton, D. (1971), *Methods Enzymol. 19,* 113–140.

Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811–816.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2,* 616–622.

Stark, G. R., and Smyth, D. J. (1963), *J. Biol. Chem. 238,* 214–226.

Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 125–140.

Wasi, S., and Hofmann, T. (1968), *Biochem. J. 106,* 926–928.