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MODIFICATION OF LYSINE AND ARGININE RESIDUES OF LYSOZYME AND THE EFFECT ON ENZYMATIC ACTIVITY

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SUMMARY

1. Acetylation of all six lysine residues of lysozyme (EC 3.2.1.17) abolished lytic action towards cells of *Micrococcus lysodeikticus* in 0.05 M phosphate buffer (pH 6.2) but did not affect cleavage of the tetramer of *N*-acetylglucosamine $(GlcNAc)_4$ obtained from chitin.

2. Acetylated lysozyme still lysed cells in solutions of low ionic strength at pH 6.2. Compared with unmodified enzyme the activity profile for acetyl lysozyme was displaced to much lower values of ionic strength and was still markedly dependent on pH.

3. Chemical modification of the lysine groups with ethyl acetamidate increased the activity towards cells slightly, yet did not alter the activity towards $(GlcNAc)_4$.

4. Modification of seven out of eleven arginine residues with 2,3-butanedione in borate buffer reduced the activity of acetyl lysozyme towards cells but not towards $(GlcNAc)_4$.

5. Since all the basic residues of lysozyme apparently lie outside the active centre, the persistence of lytic activity over a very wide pH range is discussed in terms of the currently accepted mechanism of action of lysozyme.

INTRODUCTION

It was shown in the preceding paper[1] that the lytic action of lysozyme (EC 3.2.1.17) on whole cells, and degradation of cell walls of *Micrococcus lysodeikticus*, is critically dependent on both the pH and ionic strength of the aqueous solution. In solutions of low ionic strength, lysozyme can degrade the insoluble large polymers as effectively at pH 9–10 as at pH 6. Cleavage of a small oligosaccharide from chitin, the tetramer of *N*-acetylglucosamine ($(GlcNAc)_4$), does not occur in alkaline solution but shows an optimum rate at pH 5.2.

It was proposed in earlier work[2] that basic groups of lysozyme, lying near the active centre cleft in the surface of the enzyme, might serve as proton donors in the

Abbreviation: GlcNAc, *N*-acetylglucosamine.

catalysis and provide a second hydrolytic site, thus accounting for the activity in alkaline solution. Numerous reports have appeared on the inactivation of lysozyme by a variety of reagents and procedures that affect tryptophan, methionine, histidine and lysine residues (see ref. 3). In particular, the free amino groups have been modified by acetylation with acetic anhydride[4] and by succinylation with succinic anhydride[4,5]. It has been stated that these treatments result in either complete or partial loss of enzymatic activity[4,6,7]. It thus appeared that the lysine residues were implicated, at least indirectly, in the enzymatic action. Since only Lys 97, out of six lysine residues, lies near the active centre, and at the top of the cleft as usually visualised[8], it should be possible to block its functional group completely and so destroy its possible catalytic ability. In addition some arginine residues were chemically modified and the effect on enzymatic activity was investigated.

METHODS

The source of enzyme and both types of substrate, and the assay of enzymatic activity are described in the preceding paper[1].

Acetylation

Lysozyme (2.5 mg/ml) was acetylated with various amounts of acetic anhydride (10-, 20-, 50-fold molar excess over protein) at 0° in 5 mM phosphate buffer at pH values that were varied from 6.5 to 8.0. The reaction was carried out with 4 ml of a well stirred solution using a pH-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). The pH was kept constant by the addition of 1 M NaOH and the uptake of alkali was complete after 1 h. The enzyme solution was dialysed exhaustively against 0.05 M phosphate buffer (pH 6.5), and then against water and the enzymatic activity was investigated. The extent of N-acetylation was followed by the decrease in the number of free amino groups of the enzyme, measured by the quantitative ninhydrin procedure of MOORE AND STEIN[9] and expressed as equivalents of phenylalanine. Extensively acetylated lysozyme was characterised, and for this purpose 100 mg of lysozyme were acetylated with acetic anhydride (50-fold molar excess) at pH 8.0 and the product was dialysed against water and lyophilised.

Reaction with FDNB

The number of free amino groups in native and acetyl lysozyme was determined quantitatively with FDNB. 3.5 mg of enzyme in 1 ml water were treated with twice the volume of ethanol and left for 18 h. Bicarbonate (0.2 g) and FDNB (0.1 ml) were added and the mixture shaken on a vortex mixer in the dark for 2 h. The precipitate of protein was centrifuged, washed with water (4 times), ethanol (twice) and dry ether (2 times). The DNP-proteins and samples of unreacted native and acetyl lysozyme were hydrolysed by heating in 4 M HCl in sealed evacuated tubes at 110° for 24 h. These conditions do not destroy DNP-lysine[10]. The hydrolysates were evaporated to dryness on a rotary evaporator at 37° and the residue was dissolved in 5.0 ml water. Each sample (1 ml) was diluted to 5 ml with 0.1 M HCl and the absorption spectrum was measured. ε-DNP-lysine and α, ε-bis-DNP-lysine were determined from the increase in absorbance at 364 nm over that for the unmodified proteins.

The molar absorbance coefficient ε_M at 364 nm for ε-DNP-lysine in 0.1 M HCl,

was found to be 14 500 $mole^{-1} \cdot cm^2$, and for α, ε-bis-DNP-lysine in acetone–HCl 27 700 $mole^{-1} \cdot cm^2$. In order to determine the number of DNP-lysine residues present per mole of protein, the protein concentration was determined from quantitative amino acid analysis assuming each mole of lysozyme contained 12 moles each of alanine and glycine (see below).

Amino acid analysis

Protein samples were usually hydrolysed by heating in 6 M HCl in sealed, evacuated tubes for 24 h at 110° (refluxing toluene). Quantitative amino acid analysis was performed with a Technicon Autoanalyser using 0.02 μmole of hydrolysed lysozyme and 0.25 μmole of norleucine as an internal standard. Amino acid compositions were recorded on the basis of 12.0 moles each of alanine and glycine per mole of lysozyme[11,12]. The compositions were not corrected for destruction of any amino acid during hydrolysis, and tryptophan was not determined. All modified enzymes had identical compositions other than those quoted specifically.

Digestion with trypsin

Reduction and alkylation of native and acetylated lysozyme were carried out by the method of CANFIELD AND ANFINSEN[13] adapted to a small scale (1:60). The proteins were digested with trypsin for 2 h at pH 8, 37° and the samples lyophilised. Peptide maps were obtained by chromatography of 1-mg samples in butanol–acetic acid–water (4:1:5, by vol., upper phase) for 36 h on Whatman 3MM paper, followed by high-voltage electrophoresis (40 V/cm) in pyridine–acetic acid–water (1:10:289, by vol.) buffer (pH 3.6) for 75 min (ref. 14). The peptides were detected by spraying with ninhydrin–pyridine reagent.

Amidination

Ethyl acetamidate (CH_3–C (=NH)O–CH_2–CH_3) was prepared by passing HCl through a solution of acetonitrile and ethanol[15]. Lysozyme was subjected to "partial" amidination and "exhaustive" amidination with ethyl acetamidate hydrochloride by the method of WOFSY AND SINGER[10]. Lysozyme (8 mg/ml) was made to react with 1 M reagent at pH 8.5 in borate buffer at 0° for 2 h and the excess reagent removed by exhaustive dialysis against 0.01 M phosphate buffer (pH 6.2). When assayed under the usual conditions of 0.05 M phosphate (pH 6.2, $I = 0.067$) and 0.1 M glycine (pH 9.2, $I = 0.02$) buffers the modified enzyme was 25% more active than the native enzyme.

Lysozym (70 mg) was treated exhaustively (4 lots) with acetamidate at pH 8.5–9.5 and then dialysed against borate (4 times) and phosphate (3 times) buffers and the product was again found to be a little more active towards the cell suspension than native lysozyme. The amidinated samples gave a low colour yield with ninhydrin (36% of the colour yield of native enzyme) indicating that most of the ε-NH_2 groups had reacted.

Modification of arginine residues

Native and acetyl lysozyme were treated with biacetyl (British Drug Houses) in borate buffer, according to the procedure of GROSSBERG AND PRESSMAN[16]. Reagent (1 ml) was added to 5 mg of lysozyme in 2 ml of phosphate buffer (pH 8.0). Samples (0.4 ml) were withdrawn at various times and the products separated by passage over

Sephadex G-25. The protein fractions were pooled and assayed in 0.05 M and 3.3 mM phosphate buffers (pH 6.5).

The reaction of acetyl lysozyme with biacetyl was studied in more detail, since with this protein reaction could not occur with the lysine residues. After 3 h reaction at room temperature, the solution containing 5 mg of enzyme was centrifuged and the clear supernatant solution applied to a column of Sephadex G-25 (1.5 cm × 50 cm), and eluted with 0.02 M phosphate buffer (pH 6.5). The protein emerged (volume 35 ml) just ahead of the coloured reagent and low-molecular weight products (volume >50 ml). The protein fractions were pooled, dialysed exhaustively against water (4 times 1 l), and lyophilised. Two samples of acetyl enzyme, (Samples 1 and 2, Table III), that had been treated for 3 h with reagent, which had aged for 4 and 10 days, respectively, were hydrolysed and subjected to amino acid analysis.

RESULTS

Enzymatic activity towards cell suspensions in either 0.05 M phosphate (pH 6.2) or 0.1 M glycine buffer ($I = 0.02$) (pH 9.2) was progressively lost as the molar excess of acetic anhydride over that of protein was increased from 10- to 50-fold and as the pH was raised to 8.0. However, for the partially modified enzymes, there was a change in the shape of the lytic curves compared with the behaviour of the native enzyme, and the absorbance trace was linear over approx. 40% reaction in 3 min. With a 50-fold molar excess of acetic anhydride the activity decreased to less than 1% of that of the native enzyme. The presence of a strong inhibitor of lytic activity, 0.02 M chitobiose, during the acetylation did not protect the enzyme against this loss of activity.

In addition to N-acetylation of the lysine residues, tyrosine residues were O-acetylated by this procedure with a consequent loss of absorbance at 278 nm relative to 250 nm (ref. 17). Subsequent O-deacetylation, either by treatment with alkali for 5 min (pH 12) or by reaction with 1 M NH_2OH (pH 7.5) for 15 min, of samples treated with 20- and 50-fold molar excess of acetic anhydride at pH 7.0, did not restore any activity to the enzyme, as measured under the usual assay conditions at pH 6.2 or 9.2.

The ratio of $A_{280\ nm}/A_{250\ nm}$ for the absorption spectrum of native lysozyme was 2.19–2.20 which decreased to 1.95 for acetyl lysozyme. On deacetylation of the tyrosine residues the ratio reverted exactly to that of the native enzyme, 2.20, as indicated in Table I. Since the activity was not restored on treatment with NH_2OH, modification of the tyrosine residues cannot be by itself responsible for the loss of lytic activity. In addition to the modification of tyrosine residues, there was a very small blue shift in the tryptophan absorption spectrum of lysozyme on acetylation, which has been reported recently by YAMASAKI *et al.*[18]. The extensively acetylated lysozyme (50-fold molar excess at pH 8.0, see METHODS) was essentially inactive towards cells in 0.05 M phosphate (pH 6.2). Table I shows both the number of DNP-lysine residues and the number of basic amino acid residues, determined by amino acid analysis, in the modified derivatives. Treatment with FDNB affects only the lysine, histidine and tyrosine residues; the number of each of the other amino acids was found to be unchanged. No free lysine, tyrosine or histidine was detected in the hydrolysate of the DNP derivative of native lysozyme, indicating that all these residues had reacted with FDNB. However for the acetylated lysozyme all 6 lysines and 1.4 tyrosine residues

TABLE I

CHARACTERISATION OF NATIVE AND ACETYL LYSOZYMES

Enzyme	*Ratio* $A_{280\ nm}/A_{259\ nm}$	*Ninhydrin colour (equiv Phe/mole)*	*DNP-lysine (moles/mole)*	*Amino acid residues*			
				Lys	*His*	*Tyr*	*Arg*
Native	2.19–2.20	5.6	0	5.9	1.0	3.0	10.7
Acetyl	1.90–2.0*	0.7	0	5.7	1.0	3.0	10.6
DNP-native	—	—	6.3	0	0	0	10.7
DNP-acetyl	—	—	0.0	5.8	0	1.4	10.9

* Returns to 2.20 after deacetylation with 1 M NH_2OH, pH 7.5, for 15 min.

were recovered on acid hydrolysis, clearly indicating that these residues must have been acetylated in this sample.

Since acetylation of the ε-amino groups of lysine makes the peptide bonds adjacent to these residues resistant to digestion by trypsin, cleavage is therefore limited to the peptide bonds adjacent to the 11 arginine residues. Reduced and carboxymethylated samples of acetyl and native lysozyme were digested with trypsin at pH 8.0 for 2 h to yield clear solutions. After lyophilisation, 1 mg was subjected to chromatography and electrophoresis. The peptide maps, obtained from native and acetyl lysozymes, were significantly different. The peptide map for native enzyme closely resembled that given by CANFIELD[12], and showed approx. 20 peptides, while the modified enzyme only showed 11 of these peptides. Digests of the modified protein however left some residue at the origin. For the acetyl derivative the peptide containing Lys 96 was absent (T 11, in CANFIELD's terminology), as were T 12 and T 1, free lysine. T 13 containing residues 98–112 was also absent indicating that the peptide chain was not split by trypsin between residues 97–98 or 96–97, thus supporting the conclusion that Lys 97 was acetylated in acetyl lysozyme. Earlier JOLLÈS[11] had isolated and analysed a peptide that contained residues 74–112 (T A5) from a sample of lysozyme acetylated at 0° in the presence of half-saturated sodium acetate, and this further supports the conclusion that Lys 97 can be acetylated by acetic anhydride.

Activity of acetyl lysozyme

Acetyl lysozyme was inactive towards cells of *M. lysodeikticus* under the usual assay conditions in either 0.05 M phosphate (pH 6.2) or in 0.1 M glycine (pH 9.2, $I = 0.02$) buffer, yet it still lysed the cells in solutions of very low ionic strength. Fig. 1 shows the dependence of activity on ionic strength at various pH values between 4.5 and 8.9. At pH 6.5 the activity was maximal in 3 mM phosphate buffer and amounted to 65% of the activity of the native enzyme measured in 0.05 M phosphate buffer.

Inhibition of the activity by salts (sodium chloride, phosphate and sulfate) appeared to be non-specific. The activity profile resembled that of the native enzyme but was displaced to lower values of the ionic strength. Below 5 mM phosphate buffer (pH 6.5) the acetylated enzyme was even more active than the native enzyme (see Fig. 6). Lysis of the cells became much more sensitive to changes in the ionic strength as the pH was increased from 4.5 to 8.9. Near pH 9 the acetyl enzyme was only active at extremely low concentrations of buffer, and because the pH could not be controlled

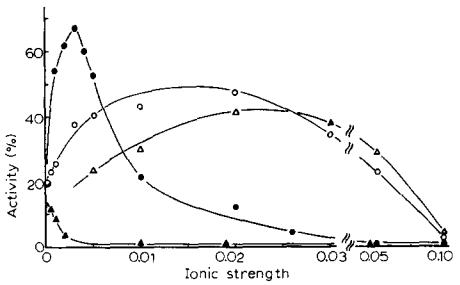


Fig. 1. Dependence of lytic activity of acetyl lysozyme on ionic strength at several pH values. Buffers: Sodium acetate–NaCl buffer, pH 4.6 (△) and pH 5.2 (○); potassium phosphate buffer, pH 6.5 (●); sodium glycinate–glycine buffer, pH 8.9 (▲). Activity is expressed as a percentage of that observed for the native enzyme in 0.05 M phosphate buffer, pH 6.2; cell suspension 78 μg/ml, 25°.

sufficiently, maximum activity was not achieved, whereas at pH 4.5 maximum activity occurred at $I = 0.025$. Consequently the observed pH optimum varied with the ionic strength of the buffer system, as was found for the native enzyme[1] (Fig. 2).

At an ionic strength of 0.05, the pH optimum for acetyl lysozyme was 4 units lower than for the unmodified enzyme. For both native and acetyl lysozymes the logarithm of the salt concentration or ionic strength that causes 50% inhibition of maximum activity varied linearly with changes in the pH of the assay, but the variation was significantly greater with acetyl lysozyme than with the unmodified enzyme (Fig. 3). Presumably this is because the total number of positive charges is less for the modified enzyme and the decreased electrostatic interaction with the cell walls is consequently more readily affected by changes in the pH.

Acetyl lysozyme remained fully active towards chitotetraose under the given

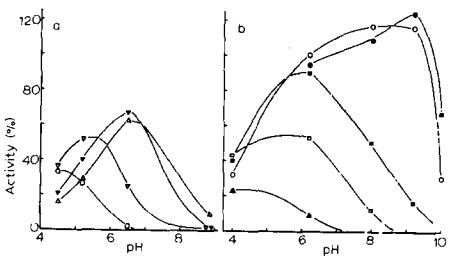


Fig. 2. Dependence of activity on pH at various values of I for (a) acetyl and (b) native lysozymes. I: 0.001 (△); 0.003 (▼); 0.01 (▽); 0.03 (●); 0.05 (○); 0.10 (■); 0.15 (□); 0.25 (▲).

assay conditions, as shown in Table II. It gave the same colour values in the Morgan–Elson reaction and the same distribution of products as did the native lysozyme. Thus acetylation of the lysine has not significantly affected the hydrolysis of the small neutral oligomer of GlcNAc. In order to test whether the binding or catalytic steps had been altered, the binding of small neutral saccharides to acetyl lysozyme was examined spectrophotometrically, with four cuvettes in tandem and enzyme 56 μM, as described earlier[2]. The acetyl and native lysozyme produced identical difference spectra in the presence of 0.05 M GlcNAc in 0.05 M phosphate buffer (pH 6.5). $\Delta\varepsilon$ (295 nm) = +680

TABLE II

LYTIC ACTIVITY AND CLEAVAGE OF $(GlcNAc)_4$ BY CHEMICALLY MODIFIED LYSOZYMES

Activity at pH 6.2 determined in 3.3 and 50 mM phosphate buffer. Activity at pH 9.2 determined in 0.1 M glycine, (I = 0.02). Enzyme activities for cell lysis and cleavage of $(GlcNAc)_4$ are expressed as a percentage of the activity of native lysozyme in 0.05 M phosphate buffer ,pH 6.2.

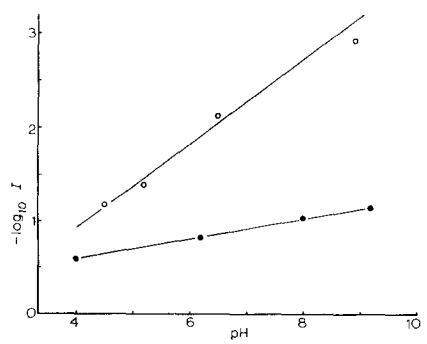
Enzyme	*Lytic activity (%)*			*Cleavage of $(GlcNAc)_4$, pH 6.2 (%)*
	pH 6.2		*pH 9.2*	
	3.3 mM	*50 mM*		
Native	46	100	120	100
Acetyl	66	1	0	100
Amidinated	72–93	125	120	90–100
Biacetyl-acetyl	14	0	0	100

$mole^{-1} \cdot cm^2$, $\Delta\varepsilon$ (267 nm) = -320 $mole^{-1} \cdot cm^2$. Acetyl lysozyme produced a very marked difference spectrum on interaction with 1 mM $(GlcNAc)_4$ in 3 mM phosphate buffer (pH 6.3), which is shown in Fig. 4, $\Delta\varepsilon$ (295 nm) = $+1480$ $mole^{-1} \cdot cm^2$, $\Delta\varepsilon$ (267 nm) = -320 $mole^{-1} \cdot cm^2$. This was similar to the difference spectrum reported by DAHLQUIST *et al.*[19] for the interaction of native lysozyme with chitotriose. Although NaCl (0.02 M) inhibited the lytic activity of the modified enzyme, it had hardly any effect on the absorption spectrum of acetyl lysozyme either in the presence or absence of $(GlcNAc)_4$.

Thus from the results reported here and in the previous paper we can conclude that the hydrolysis and the binding of neutral substrates, as reflected by the perturbation of the tryptophan chromophores of lysozyme by GlcNAc and $(GlcNAc)_4$, are not affected by either modification of the lysine residues or by the presence of appreciable concentrations of salt.

Further modification of lysozyme

The conversion of lysine into homoarginine residues was followed by the reaction



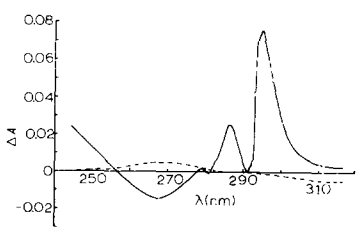


Fig. 3. Correlation of log I or salt concentration causing 50% inhibition of maximum activity, with pH of the assay, for native (●) and acetyl (○) lysozymes. The upper point for acetyl lysozyme represents the upper limit for 50% inhibition, $I = 1.3 \cdot 10^{-3}$.

Fig. 4. Difference spectra of acetyl lysozyme with $(GlcNAc)_4$ (1 mM) (———); and with NaCl (0.21 M) (- - - - - -). Acetyl lysozyme, 51.4 μM, in 3 mM phosphate buffer ,pH 6.3, 25°. Difference spectra of samples were recorded with acetyl lysozyme in the reference cuvette.

of the free amino groups with ninhydrin and did not go to completion with lysozyme, even under the conditions of "exhaustive amidination"[10]. The amidinated product was still fully active towards $(GlcNAc)_4$ at pH 5.5, $I = 0.15$, and lysed cells even in 0.05 M phosphate buffer (pH 6.2). However, the modified enzyme was 120% as active as the native enzyme at pH 6.2 and 9.2, and the lytic activity showed a dependence on salt concentration similar to that observed with the native lysozyme.

Arginine and lysine residues in proteins can be modified chemically by several diketo reagents[16,20-22], of which 2,3-butanedione (biacetyl) appears to be most effective under mild reaction conditions. The presumable active reagent of "biacetyl" solutions in borate is a trimer[23] which reacts very effectively with arginine residues, but the precise nature of the reaction products is not yet known.

Treatment of acetyl lysozyme with biacetyl for 3 h, only affected the arginine residues, as determined from amino acid analysis. As the arginines were modified there was a concomitant decrease in the lytic activity measured in 3 mM phosphate buffer (Table III). Samples of these modified enzymes, however, were still fully active towards

TABLE III

ACTIVITY AND ARGININE CONTENT OF LYSOZYMES TREATED WITH BIACETYL

Lytic activity was measured in phosphate buffers, 3 and 1 mM. Activities are expressed as a percentage of the activity of acetyl lysozyme in 3 mM phosphate buffer for lysis, and in 0.05 M phosphate buffer, pH 6.2, for cleavage of $(GlcNAc)_4$.

Enzyme	*Lytic activity (%), pH 6.5*		*Cleavage of $(GlcNAc)_4$ (%)*	*Residues of Arg (moles/mole)*	*Arg modified (moles/mole)*
	3 mM	*1 mM*			
Native	76	30	100	10.7	
Acetyl	100	80	100	10.6	
Sample 2*	62	—	—	6.1	4.5
Sample 1	35	50	100	4.0	6.6

* Samples 1 and 2 were acetyl lysozyme treated with biacetyl for 3 h (see METHODS).

$(GlcNAc)_4$ (3 mg/ml) in 0.04 M phosphate buffer (pH 6.2), and in fact the μg equivalents of GlcNAc formed during the assay increased linearly with time over 24 h and coincided exactly with the amounts formed by both native and acetyl lysozymes.

Acetyl lysozyme was inactivated by biacetyl much faster than native lysozyme which had six more lysines available for reaction as well as arginine (Fig. 5). Samples of the modified enzyme that had been exposed to biacetyl for 5–6 h were examined and found to have an altered dependence of activity on ionic strength. Native lysozyme treated with biacetyl showed maximum activity in 0.01 M phosphate buffer, but was only 25% as active as native lysozyme. Acetyl lysozyme treated with biacetyl had maximum activity in 1 mM phosphate buffer and this maximum only amounted to 15% that of the native enzyme measured in 0.05 M phosphate buffer (pH 6.2). However, the activity was only slightly less than the activity of the native enzyme if this was assayed at the same ionic strength (see Fig. 6).

Thus again the activity maximum has been decreased and moved to very low values of the ionic strength as the basic residues were modified, whilst the activity

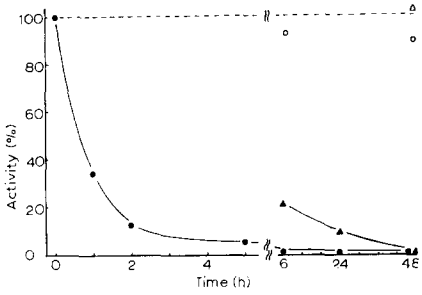


Fig. 5. Reduction in activity of native (▲) and acetyl lysozyme (●) in borate buffer, pH 8.2, on reaction with biacetyl. Closed symbols, samples; open symbols, controls run in the absence of reagent. Enzymes were assayed in 3 mM phosphate buffer, pH 6.5, and activities of untreated samples at time zero were taken as 100%. Samples of native enzyme treated with reagent for 6 h were inactive in 0.05 M phosphate buffer.

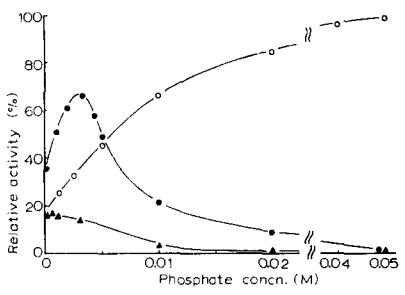


Fig. 6. Relative activity of modified lysozymes in phosphate buffers, pH 6.5. Native lysozyme (○); acetyl lysozyme (●); an acetyl lysozyme treated with biacetyl for 3 h (▲). Activity is expressed relative to native enzyme assayed in 0.05 M phosphate, pH 6.5.

towards chitotetraose has not been affected. As was found for the acetyl lysozyme (Fig. 1), the ionic strength optimum moved to higher values as the pH of the assay was lowered, *i.e.*, from $I = 0.001$ at pH 6.5 to $I = 0.15$ at pH 4.5, and the observed pH and ionic strength optima for lysis were not independent of each other.

DISCUSSION

The importance of basic groups in the enzymatic function of lysozyme

Acetyl lysozyme used in these studies had apparently all the lysine groups acetylated and protected against reaction with FDNB. Reaction of native lysozyme with FDNB went very nearly to completion (Table I). The slight discrepancy between the observed and theoretical number of DNP-lysine residues and yet the absence of any free lysine on hydrolysis suggests that, if any residue were only partially modified, it was probably the N-terminal lysine and not Lys[97]. Comparison of the peptide maps of trypsin digests of the native and acetylated proteins in fact showed in particular that Lys 96 and Lys 97 must have been acetylated in the modified enzyme.

GlcNAc and $(GlcNAc)_4$ perturbed the indole chromophores of the tryptophan residues, and the native and modified enzymes were affected to the same extent, irrespectively of the salt concentration. The small substrate, $(GlcNAc)_4$, was hydrolysed at the same rate by lysozyme and all its modified derivatives and produced the same cleavage pattern. It must therefore be concluded that the positively charged lysine and arginine residues, including Lys 97, do not participate either in the binding of these small molecules or in the catalytic step. It therefore follows that they lie outside the active centre. This is in agreement with the crystallographic findings of BLAKE *et al.*[8].

The apparent loss of lytic activity of lysozyme by acetylation results from the assays being conducted at relatively high pH and high ionic strength. If lysis had been examined in 3 mM phosphate buffer (pH 6.2) for example, the activity of lysozyme would have shown an increase on acetylation. A reduction in the number of positive charges on lysozyme displaces the activity maximum to lower values of ionic strength

at constant pH, and to lower pH values at constant ionic strength. Consequently, the direct participation of specific amino acid residues in enzymatic catalysis cannot be deduced from observations made under one particular set of assay conditions. While this work was being completed other reports of modification of lysine and alteration of enzymatic activity have been made. MARZOTTO *et al.*[24] modified the lysine residues by acetoacetylation with diketene and found decreased lytic activity; both processes could be reversed by treatment with NH_2OH. HAYASHI and co-workers[18,25,37] reported that acetylation of lysozyme in half-saturated sodium acetate did not affect enzymatic activity towards glycol–chitin at pH 5.5, but abolished lytic activity at pH 6. Modified enzymes showed pH optima for lysis of whole cells and degradation of cell walls that differed from that of the native enzyme; and this is confirmed by our data. However the cause of the shift lies in alterations of the dependence of activity on ionic strength as well as on pH. Minor changes in the conformation of the protein on acetylation were suggested by a small spectrophotometric perturbation of the tryptophan residues and an increased rate of digestion of the enzyme by several proteases, but no major changes occurred in the conformation of the main peptide chain as determined by optical rotatory dispersion.

Modification of seven of the eleven arginine residues in acetyl lysozyme with biacetyl did not affect the cleavage of $(GlcNAc)_4$. Although X-ray crystallography had suggested the possible involvement of Arg 114 in binding $(GlcNAc)_6$ to site F^6, no residue of arginine possibly involved in catalysis had apparently been modified in our experiments. As was observed for the acetylation of lysozyme, removal of more positive charges from the enzyme displaced the activity maximum to very low values of ionic strength.

Conversion of lysine residues into more basic homoarginine residues by amidination with ethyl acetamidate, as reported here, or with long chain amidines[26] or by guanidination with *O*-methyl isourea[6], does not alter the overall charge on the protein. We found that amidination did not alter either the enzymatic activities or the effects of pH and ionic strength in agreement with this conclusion. Proteolytic cleavage of lysylvalyl residues from the N-terminus[27] or arginylleucine from the carboxyl terminus[28] of lysozyme were reported to give derivatives that are still enzymatically active. Both are slightly less basic than native lysozyme and in agreement with the observations reported here, the des-arginylleucine enzyme had an activity profile that was displaced to slightly lower values of ionic strength.

In solutions of relatively high ionic strength the pH optima for lysis and for cleavage of neutral oligosaccharides derived from chitin by native and modified enzymes become more similar, *i.e.*, are in the range of pH 4–5 (see also glycol chitin[18,29,37]). It must be presumed that under these conditions the electrostatic interaction between lysozyme and cell wall now becomes of only secondary importance in determining the overall rate of cleavage. In addition, however, as the Coulomb interaction with the cell wall is decreased, either by increasing the ionic strength or by reducing the positive charge on the enzyme, the maximum lytic activity attained becomes smaller (Fig. 2).

Mechanism of action over a wide pH range

The persistence of lytic activity for native lysozyme over the pH range 4–10 and for fully acetylated lysozyme over the range 4–9, although at greatly different values

of ionic strength, suggests that the mechanism of lysis is not affected by modification of the lysine groups and is probably the same at pH 9 as at pH 6.

On the basis of the crystallographic data of BLAKE *et al.*[8] all enzymatic catalysis is considered to occur within the cleft and the proposed proton transfer mechanism[30] involving Glu 35 is considered to be the most plausible scheme at present, although no direct evidence for it is yet available. It is difficult to find a proton donor in the cleft other than a carboxyl group since Lys 97 has now been excluded. Thus we must consider the possibility that the carboxyl group of Glu 35 is partly un-ionised in solutions of pH 10 when combined with certain high molecular weight substrates.

The Glu 35 residue lies in a hydrophobic part of the cleft and has been assigned a pK value somewhat higher than usually found for carboxyl groups in proteins, *i.e.*, 3.5–5.0. On formation of an enzyme–saccharide complex there is a further apparent increase in the pK to a value well above 6.0 (refs. 19, 31, 32) and the catalytic group is presumably now in a more hydrophobic environment in which the effective "dielectric constant" has been lowered, perhaps by the exclusion of water molecules from the cleft. However to account for the high rate of cell lysis by lysozyme at pH 9–10, the pK of the carboxyl group under the assay conditions must be appreciably higher than 6–7.

It seems likely therefore, that when lysozyme is strongly adsorbed onto an insoluble charged substrate, such as a cell wall, by suitable combinations of ionic strength and pH of the bulk solution, the effective dielectric constant around the catalytic group is further lowered, and the solvation and effective ionisation of the carboxylate group is consequently greatly reduced.

The pH of the microenvironment around the active centre of enzymes which are attached to solid supports may indeed differ from the pH of the bulk solution, and this has been discussed by KATCHALSKI and co-workers[33,34], *e.g.*, a shift of 2.0–2.5 units was observed for reactions catalysed by charged solid-phase derivatives of trypsin. Marked effects of pH and ionic strength, which are greater than those found for homogeneous reactions, have also been reported for several heterogeneous reactions[35,36].

The occurrence of enzymatic activity in alkaline solution and a similar apparent shift in pK of the catalytic group has not yet been demonstrated for interactions of lysozyme with large charged soluble substrates, but in general the same considerations, as discussed above, will apply. It would thus appear that the findings reported in this and the earlier paper are not necessarily at variance with the mechanism proposed by BLAKE *et al.*[8] and by VERNON[30].

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