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Histidine-15 and lytic activity of lysozyme

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Abstract. The literature data on the activity of histidine-15 modified hen egg white lysozyme are conflicting: the modified enzyme is reported to have more activity, similar activity or less activity by different authors. Amino acid analysis had shown modification of the single His-15. Detailed activity studies on His-15-modified (by iodoacetic acid or diethyl pyrocarbonate) lysozyme have shown that the contradicting reports are due to the specific choices of ionic strengths and cell wall substrate concentrations and can be attributed to the substrate being negatively charged. Our analysis suggests that even though histidine-15 is far removed from the active site of lysozyme, its chemical modification or binding of the negatively-charged substrate near it, changes the conformation around the active site. However, the change in the optimum activity on chemically modifying His-15 is small.

Keywords. Lysozyme; histidine modification; *Micrococcus lysodeikticus*; electrostatic potential in catalysis; pK-shifts.

1. Introduction

In the recent past, there has been considerable interest in the role played by regions far removed from the active site of enzyme in the substrate binding and catalysed reactions (Krishnamoorthy *et al* 1979; Grütter and Mathews 1982; Krishnamoorthy and Prabhananda 1982; Veerapandian *et al* 1985). His-15 of hen egg white (HEW) lysozyme is remote from the active site (Blake *et al* 1965; Jolles *et al* 1965). Lysozymes in which His-15 is not present also show lytic activity towards. *Micrococcus lysodeikticus* cell walls (*M. luteus*) (Jolles *et al* 1965; Imoto *et al* 1977). Therefore, His-15 does not seem to play a direct role in controlling the lytic activity.

However, reduced activity (~ 66%, taking the activity of native enzyme to be 100%) has been reported for lysozyme in which spin labels had been covalently and specifically linked to His-15 (Wein *et al* 1972). A comparison of X-ray crystallographic data and the distance estimates made with the help of magnetic relaxation studies, had confirmed the location of spin labels on His-15 (Wein *et al* 1972). Therefore, it would appear that changes in the region around His-15 could change the lysozyme activity, at least indirectly.

Conflicting reports have appeared in literature about the activity of lysozyme in

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Abbreviations used: HEW lysozyme, hen egg white lysozyme; CE lysozyme, carbethoxyhistidine-15 lysozyme; CM lysozyme, 3-carboxymethylhistidine-l5 lysozyme: DPC, diethylpyrocarbonate; *M. luteus*, *Micrococcus lysodeikticus,* Bistris, bis [2-hydroxyethyl] iminotris [hydroxymethyl] methane; Mes, 2-[N-morpholino] ethanesulfonic acid.

which His-15 is modified with iodoacetic acid. Kravchenko *et al* (1963) have reported increased activity (110–112%) when only His-15 had been modified. In contrast with this report, Piszkiewicz and Bruice (1968) confirmed Hartdgen's (1967) observation of a decrease in lytic activity (40–45%) on carboxymethylation of His-15. On the other hand, Parsons *et al* (1969) did not observe any change in the activity.

Since the lysozyme activity towards cell wall substrate is known to be ionic strength-dependent (Davis *et al* 1969; Maurel and Douzou 1976), one can expect the explanation of the conflicting results reported in literature to be based on the specific choice of ionic strength in the experiments. The present studies were undertaken to examine such a possibility and to find a rational explanation for the above mentioned contradicting results. Two types of chemical modifications of His-15 have been used for this purpose, (i) Carboxymethylation of His-15 by the procedure of Parsons *et al* (1969) using iodoacetic acid, (ii) Carbethoxylation of His-15 by the reaction of lysozyme with diethyl pyrocarbonate (DPC) (Miles 1977). The two types of chemical modifications enable us to look for the differences in the activity behaviours when the charges on the chemically modified His-15 are different. Apart from clarifying the conflicting reports, our present studies have shown the importance of His-15 in the regulation of activity towards *M. luteus* cell walls.

2. Materials and methods

HEW lysozyme, DPC and *M. luteus* were obtained from Sigma (USA), iodoacetic acid from Aldrich (USA). Carbethoxyhistidine-15 lysozyme (CE lysozyme) was prepared by adding 8 μl of 250 mM DPC in ethyl alcohol to 1 ml of 1 mM HEW lysozyme in 50 mM Bistris buffer at pH 5·5. The reaction mixture was dialyzed against 10 mM Bistris at pH 7, after allowing the reaction to proceed for about 1 h at 24°C. CE lysozyme had reduced activity (~65% at pH 7·0 and $[KNO_3]$ = 0·1 M). Chemical modification of His-15 of lysozyme was inferred by noting the presence of one carbethoxyhistidyl residue per enzyme from the optical density ($\Delta\varepsilon_{240}$=3200 M^{-1} cm^{-1}) (Miles 1977) and from the reversal of the modification by treatment with hydroxylamine (9 ml of 1 M NH_2OH HCl added to 1 ml of 1 mM CE lysozyme) for about 10 min which restored the activity to 100% level (Miles 1977).

The 3-carboxymethylhistidine-15 lysozyme (CM lysozyme) was prepared by a procedure similar to that of Parsons *et al* (1969). A reaction mixture prepared by dissolving 0·18 g of lysozyme and 1·3 g of iodoacetate in 10 ml of 0·1 M acetate buffer at pH 5·5 was kept at 40°C for 22 h. Subsequently, it was dialyzed against 10 mM Bistris at pH 7·0. The chromotographic analyses using Bio-Rex 70 and the lytic activity data at μ = 0·1 and pH = 7 agreed with the literature data (Piskiewicz and Bruice 1968; Hartdgen 1967) and suggested that we had > 90% of lysozyme modified to CM lysozyme in our preparations.

Lytic activity towards *M. luteus* (Salton 1952; Salton and Pavlik 1960) was measured in 20 mM Bistris buffer (pH = 7) as described elsewhere (Krishnamoorthy *et al* 1979; Krishnamoorthy and Prabhananda 1982). The activity at a given experimental condition was determined from at least four measurements. The

variation in the activity measured in the four measurements was < 6%. The trends observed were confirmed by duplicating the experiments with a second set of stock solutions. Experiments were also carried out using KCl to regulate the ionic strength and at other pH conditions to confirm that the observed behaviours were not due to the specific choices of electrolyte or pH.

3. Results

The chemical modifications of lysozyme described in this work were carried out under conditions such that His-15 was specifically altered. Reaction with DPC under our conditions generated only one carbethoxyhistidyl residue per molecule of the enzyme as shown by its optical absorption at 240 nm (Miles 1977), and the restoration of activity to the 100% level by hydroxylamine treatment (Miles 1977). Reaction with iodoacetic acid was carried out under conditions similar to those described by Parsons *et al* (1969) who had shown by amino acid analysis the specific reaction at the single histidine residue.

The hypothesis that the differences in the experimental conditions used by different authors [which are not explicitly mentioned in the literature (Kravchenko *et al* 1963; Parsons *et al* 1969)] could be responsible for the conflicting reports, was confirmed by studying the lytic activities at different ionic strengths and substrate concentrations as shown below.

At low ionic strengths, the activity of CE lysozyme is higher than that of HEW lysozyme at all the substrate concentrations used in our experiments (figures 1 and 2). However, the activity of CM lysozyme at low ionic strengths is close to that of HEW lysozyme at low substrate concentrations (figures 1a and 2a), but higher than that of HEW lysozyme at higher substrate concentrations (figures 1b and 2b). These observations are well beyond the experimental errors and reproduce the behaviours reported by Kravchenko *et al* (1963) or Parsons *et al* (1969) by a suitable choice of the experimental condition. On the other hand, the high ionic strength data (figures 1 and 3) show that the activities of CE and CM lysozymes are less than that of HEW lysozyme which is similar to that reported at ionic strength 0·1 (Piskiewicz and Bruice 1968; Hartdgen 1967).

The shifts in the ionic strengths of maximum activity on chemical modification seen in figure 1 were also seen in the studies carried out under other pH conditions (eg. pH 5·8 and 9·8). At a given ionic strength, the pH of maximum activity showed a shift towards smaller value on chemically modifying His-15. Typical data obtained in such studies are given in figure 4.

4. Discussion

In the mechanism of lysozyme action, k_{cat} is determined by the probability that Glu-35 is protonated and Asp-52 is deprotonated (Imoto *et al* 1977). Experimentally observed pH dependence profile of lysozyme activity towards *M. luteus* is ionic strength-dependent (Davis *et al* 1969; Maurel and Douzou 1976). Maurel and Douzou (1976) could explain such a behaviour on the basis of pH shifts due to electrostatic potential, ψ, from the negatively-charged cell wall substrate

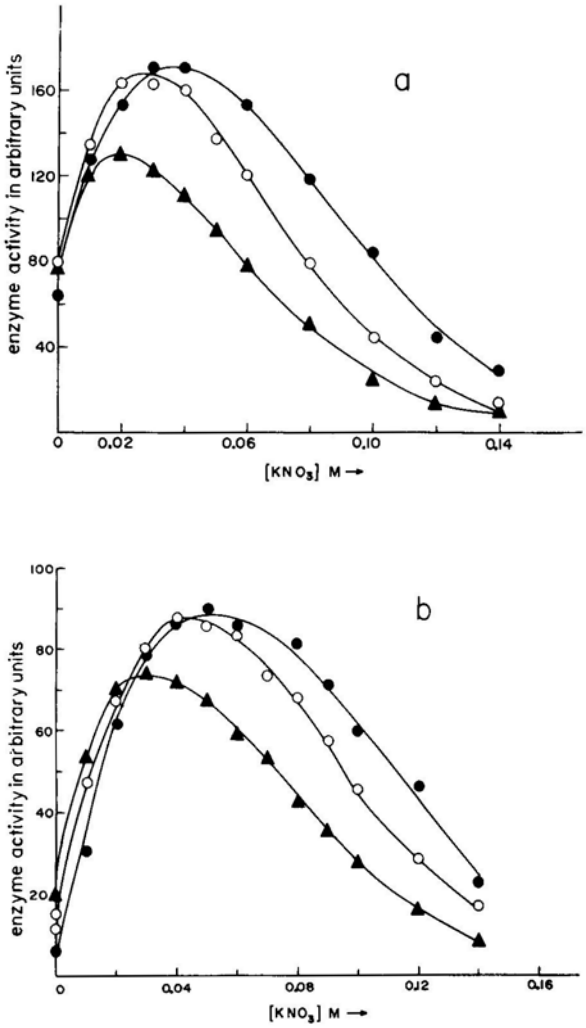

Figure 1. Dependence of activity on ionic strength for HEW lysozyme,(●) CE-lysozyme (O), and CM-lysozyme (▲). *M. luteus* concentration **(a)** 0·062 mg/ml, **(b)** 0·12mg/ml. $[E]_0 = 3 \times 10^{-8}$M **(a)** 2×10^{-8}M **(b)** pH = 7·0 and temperature 24°C. Buffer concentration was 20 mM Bistris. Activity values are averages of at least four measurements. The variations were within 6%.

bound to enzyme. The negative charge on the substrate is associated with the peptide fragment that cross-links polysaccharide chains.

$$pH_{in} = pH_{out} + \varepsilon\psi/2{\cdot}303\ kT, \tag{1}$$

where pH_{in} and pH_{out} are the local and bulk values of pH respectively (Maurel and Douzou 1976). Only pH_{out} is experimentally accessible. ε is the unit charge. A similar equation would be applicable for the apparent pK values of Glu-35 and Asp-52.

$$PK_{app} = pK_0 - \varepsilon\psi/2{\cdot}303\ kT \tag{2}$$

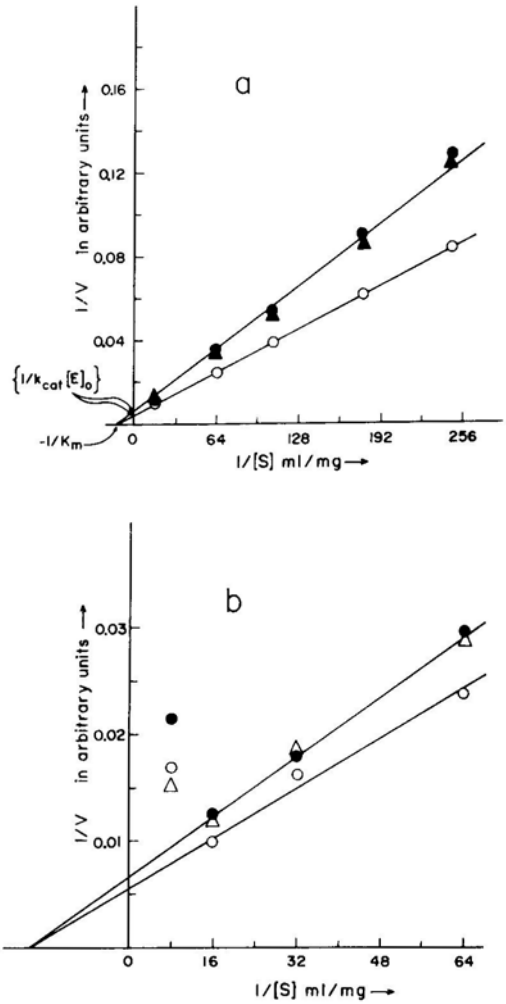

Figure 2. Lineweaver-Burk plots for HEW lysozyme (●), CE-lysozyme(O) and CM-lysozyme (▲), **(a)** $[KNO_3]$ = 0·007 M, $[E]_0 = 3 \times 10^{-8}$ M, pH = 7·0 and temperature 24°C. **(b)** Deviation from the straight line behaviour of Lineweaver-Burk plots seen at low ionic strengths for HEW lysozyme(●) CE-lysozyme (O) and CM-lysozyme (Δ). $[E]_0 = 3 \times 10^{-8}$ M, $[KNO_3]$ =0M, and pH = 7·0. Buffer concentration was 20 mM Bistris.

Since is negative, $pK_{app} > pK_0$ where pK_0 is the value in the absence of ψ. The screening of the electrostatic potential, ψ, and hence the pK-shifts, could be varied by varying the ionic strengths (I) of the solutions.

$$\psi = A - B \log (I), \tag{3}$$

where A and B are constants (Maurel and Douzou 1976). In this model, at a given pH, optimum activity is obtained at an ionic strength at which the average of pK_{app} values of Glu-35 and Asp-52 is equal to the pH. At other conditions, k_{cat} will have reduced values.

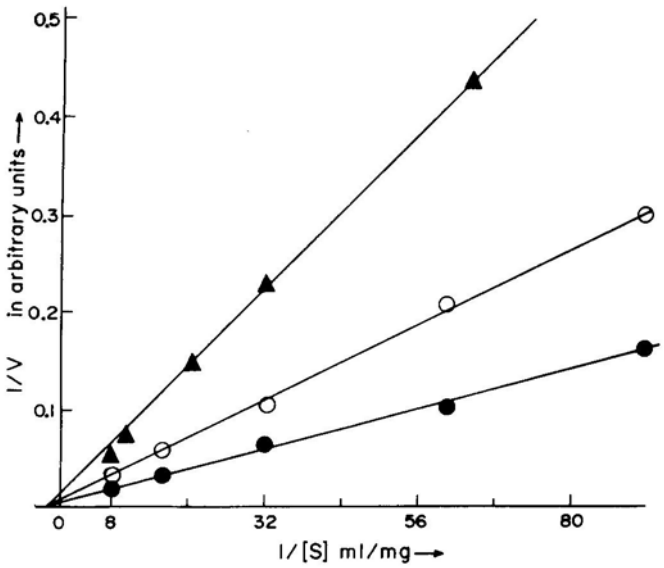

Figure 3. Lineweaver-Burk plots for HEW Lysozyme (●), CE-lysozyme (O) and CM-lysozyme (▲), at high ionic strength. $[KNO_3]$ = 0·14 M, $[E]_0 = 3 \times 10^{-8}$ M, pH = 7·0 and temperature 24°C. Buffer concentration was 20 mM Bistris.

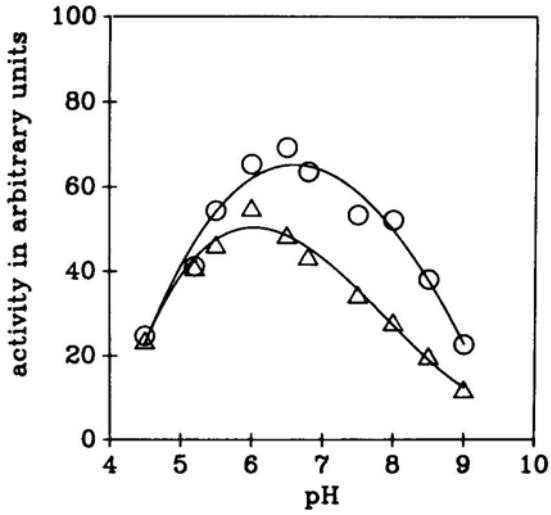

Figure 4. Dependence of activity of HEW lysozyme (O) and CM-lysozyme(Δ) on pH at ionic strength 0·1. The ionic strength was regulated using KC1. The buffers used were sodium acetate (pH 4·5-5·5), Mes (pH 5·5-7·0) and Tris (pH 7·5-9·0).

According to this model, if the negatively-charged groups of the cell wall substrate bind at a greater distance from the active site of lysozyme on chemically modifying His-15 (hence requiring smaller ion concentrations to screen the electrostatic potential to get appropriate *pK* shifts), (i) the ionic strength of

optimum activity shifts to smaller values, (ii) the differences in activities of the enzyme preparations observed at low and high ionic strengths will be dominantly due to differences in k_{cat} and (iii) the pH of optimum activity would shift towards a smaller value. These three predictions are observed in the data of figures 1–4. The data in figures 1 and 2 show clearly that the conflicting reports (Kravchenko *et al* 1963; Piskiewicz and Bruice 1968; Hartdgen 1967; Parsons *et al* 1969) on the effect of modification of His-15 could be due to different experimental conditions (pH, ionic strength and substrate concentration) employed during the assay of enzyme activity. The data also suggest a conformational change near the active site of lysozyme on chemically modifying His-15. Furthermore, a larger change in the conformation near the active site, inferred from the shift in the ionic strength of maximum activity in the case of CM lysozyme (figure 1), is also accompanied by a decrease in the maximum $k_{cat.}$ A parallel situation is the correlation observed between the inactivation due to oxidation of Trp-108 and the displacement of Glu-35 observed in X-ray studies (Blake *et al* 1967).

At low ionic strengths and at high substrate concentrations, the velocity of product formation decreases with increase in substrate concentrations (figure 2b), suggesting the possibility of substrate inhibition of lysis. Such behaviours at low ionic strengths have also been observed by Verhamme *et al* (1988) in the case of HEW lysozyme. The results were best understood in a model (Verhamme *et al* 1988) in which a second substrate binds to the enzyme-substrate complex (*ES*) leading to an inactive complex (ES_2). The rate of lysis, *V*, is then given by (Verhamme *et al* 1988).

$$\frac{1}{V} = \frac{1}{k_{cat}[E]_0}\left[1 + \frac{K_m}{[S]} + \frac{[S]}{K_2}\right], \tag{4}$$

where K_m is the Michaelis constant and $K_2 = [ES]\cdot[S]/[ES_2]$. The third term explains the "deviations" from the straight line behaviour seen in figure 2b The smaller deviation observed for CM lysozyme (figure 2b) implies smaller stability for the second substrate binding in the case of CM lysozyme when compared to in HEW and CE lysozymes. Such an observation at lower ionic strengths could be understood by assigning the second substrate binding site to a region around His-15, whose net positive charge (Blake *et al* 1965; Imoto *et al* 1977) (which favours the binding of negatively-charged cell wall substrates) is reduced by the carboxymethylation of His-15. From carbon-13 NMR studies, Goux and Allerhand (1979) concluded that chemical modification of His-15 (as in CM lysozyme) does not significantly change the *pK* (~ 5·5) associated with His-15. Therefore, at pH 7·0 we have a negligible net charge on His-15 in HEW and CE lysozymes but a net negative charge on modified His-15 in CM lysozyme. The electrostatic potential due to the cell wall substrate at His-15 in the enzyme-substrate complex, inferred from inhibition studies (Krishnamoorthy and Prabhananda 1982), provides further support for the suggestion that the peptide fragments of *M. luteus* bind near His-15. Since negatively-charged species have been known to bind in the region around His-15 (Imoto *et al* 1977; Blake 1968), the above suggestion is reasonable. If there could be conformation changes at the far away active site on chemically modifying His-15 (inferred above) reducing the enzyme activity, the binding in the region around His-15 could also reduce enzyme activity, making ES_2 inactive as required by the model (Verhamme *et al* 1988). It is hoped this study will help in recognizing

the possibility of such changes in the activity even in the case of other enzymes, when charged substrates are involved.

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