J. Biosci.,Vol. 5, Number 4, December 1983, pp. 331–338.

Interaction of lysozyme with antibiotics –binding of penicillins to lysozyme

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MS received 10 October 1983; revised 25 October 1983

Abstract. Binding of lysozyme with the antibiotics such as penicillin-G, penicillin-V and methicillin at different concentrations and pH was studied by equilibrium dialysis. Co-operative binding isotherms were observed at pH 5.0,7.0 and 9.0 with all the penicillins and the binding ratios decreased slightly with the increase of pH. The Gibbs free energy change calculated on the basis of Wyman's binding potential concept decreased slightly with the increase of pH indicating slight decrease in the binding strength at higher pH in the case of all penicillins.

The ultra-violet difference spectra of lysozyme-penicillin complexes showed a less intense peak in the region of 284-300 nm at pH 5.0. Only penicillin-G complex had a peak at pH 7.0 at these wavelengths with less intensity compared to that at pH 5.0. However, none of the penicillins showed discrete peaks in this region at pH 9.0. The appearance of peaks in the difference spectra of all these complexes at pH 5.0 and with only penicllin-G complex at pH 7.0 in the aromatic region indicated hydrophobic interactions with tryptophan residues as the binding sites. In addition, the ionic interactions with lysine residues in lysozyme were also occurring.

The conformational changes induced by the binding of penicillins to lysozyme monitored by circular dichroism showed a slight decrease in the aromatic bands in the 320-250 nm region. However, in the 250-200 nm region, $[\theta]_{222nm}$ values obtained at various concentrations of penicillins in the complex indicated an increased α- helical content generating a more ordered structure. These results led to the conclusion that both the hydrophobic and electrostatic interactions prevail in the binding of penicillins to lysozyme.

Keywords. Lysozyme; penicillins; co-operative binding; helical content.

Introduction

A study of the binding of penicillins to proteins is important in the understanding of its function in absorption, transport, anti-bacterial activity, toxicity etc. The reversible binding (Klotz *et al.*, 1950; Rolinson and Sutherland, 1965) of penicillins and other drugs to proteins demonstrated using ultrafiltration (Keen, 1965, 1966; Bird and Marshall, 1967) *in vitro* and *in vivo* has been a subject of extensive discussion on their probable significance. However, the exact nature of the chemical interactions and the binding sites involved were not clearly identified. Ionic binding of penicillins to lysozyme has been studied in detail by pulse radiolysis (Philips *et al.*, 1970, 1973), proton magnetic resonance (Mitsumori *et al.*, 1980), fluorimetric and ultra-violet (UV) difference spectroscopy (Felsenfeld and

Abbreviations used: CD, Circular dichroism; UV, ultra-violet.

Handschumacher, 1967), x-ray diffraction technique (Johnson, 1967), radio isotope labelling (Corran and Waley, 1975) and equilibrium dialysis (Klotz *et al.*, 1950). These studies reveal that the side chains of penicillins are involved in the hydrophobic interactions especially with tryptophans in the protein, while the carboxylate anionic groups in penicillins bind at the cationic sites such as lysines. The stoichiometry reported for lysozyme-penicillin complexes from these studies is found to vary with the type of penicillin which basically differs in the hydrophobic character of R group as well as the nature and number of cationic sites available for binding through electrostatic interactions.

In the present investigation lysozyme was chosen as a model protein because its three dimensional structure, its physico-chemical characteristics and its biological function were extensively studied (Johnson and Philips, 1965; Rupley, 1967; Rupley and Gates, 1967; Sophianopoulos and Van Holde, 1964; Sophianopoulos and Weiss, 1964; Blake *et al.*, 1965,1967; Philips, 1966). The interaction of lysozyme with penicillins such as penicillin-G (benzyl penicillin), penicillin-V (Phenoxy methyl penicillin), and methicillin (2,6-dimethoxy phenyl penicillin) at various concentrations and pH, using equilibrium dialysis technique are described here. The thermodynamic parameters of binding were evaluated. The nature of the binding sites and the interactions there at was determined by UV difference spectra, and the conformational changes accompanying the binding were followed by circular dichroism (CD) technique. However, the enzymatic activity of lysozyme due to penicillin binding was not studied.

Materials and methods

Hen egg white lysozyme (3X crystallized, lot No. 6876), penicillin-G, penicillin-V and methicillin obtained from Sigma Chemical Company, St. Louis, Missouri, USA were used. Imidazole (British Drug House, Poole, England) was recrystallized thrice from benzene to remove UV absorbing impurities. All other reagents used were of analytical reagent grade and the solutions were prepared using double distilled water. For equilibrium dialysis, membrane tubing from Union Carbide Corporation, Chicago, Illinois, USA, was used. UV-Visible spectrophotometer (Carl Zeiss) was used to measure the concentration of lysozyme and penicillins. $E^{1\%}_{280cm}$=26.4 for lysozyme (Sophianopoulos *et al.*,1962) was used to calculate the concentration of lysozyme.

The concentrations of penicillins are determined by absorbance measurements at 325–335 nm of penicillinic acid mercuric mercaptide of the respective penicillins formed in quantitative yield on heating at 60° C with 1.2 M imidazole and 1 mM mercuric chloride solution as described elsewhere (Bundgaard and Ilver, 1972).

Jasco J-20 spectropolarimeter previously calibrated with (+)-10 camphor sulphonic acid (De Tar, 1969) was used to record the CD spectra. Beckmann spectrophotometer Model-25 was used to record the UV difference spectra.

In the equilibrium dialysis experiments, 5 ml of 0.2% lysozyme (0.14 mM) was dialysed against 10 ml of the penicillin solution at various concentrations in the same buffer medium as described earlier (Subramanian and Venkatappa, 1982). The time required for equilibration was found to be o h at 25 °C. The binding ratios *'r'*, the number of mol of penicillin bound per mol of the protein at various

free penicillin concentrations [D], are calculated in a way similar to that of Rosenberg and Klotz (1961) .

The buffers used were; 0.01 M sodium acetate-acetic acid buffer, pH 5.0,0.01 M N a_2 H P O_4 - KH $_2$ PO $_2$ buffer, pH 7.0 and 0.01 M glycine buffer, pH 9.0. The binding studies were carried out using 0.14 mM lysozyme and varying the penicillin concentration (1–7 mM), while the UV difference spectra was recorded at 0.07 mM enzyme concentration.

The CD spectra of lysozyme at 23.5 and 35 µM concentrations and its complex with penicillin were measured at 25° C in a 0.5 mm cell in the 250-200 nm region and using a 10 mm cell in the 320-250 nm region respectively. The results are represented as molar residue ellipticity,$[\theta]_\lambda$ in deg cm^2 $dmol^{-1}$,calculated in a manner similar to that of Adler *et al.* (1973) taking a value of 111 as mean residue weight for lysozyme (Warren and Gordon, 1970).

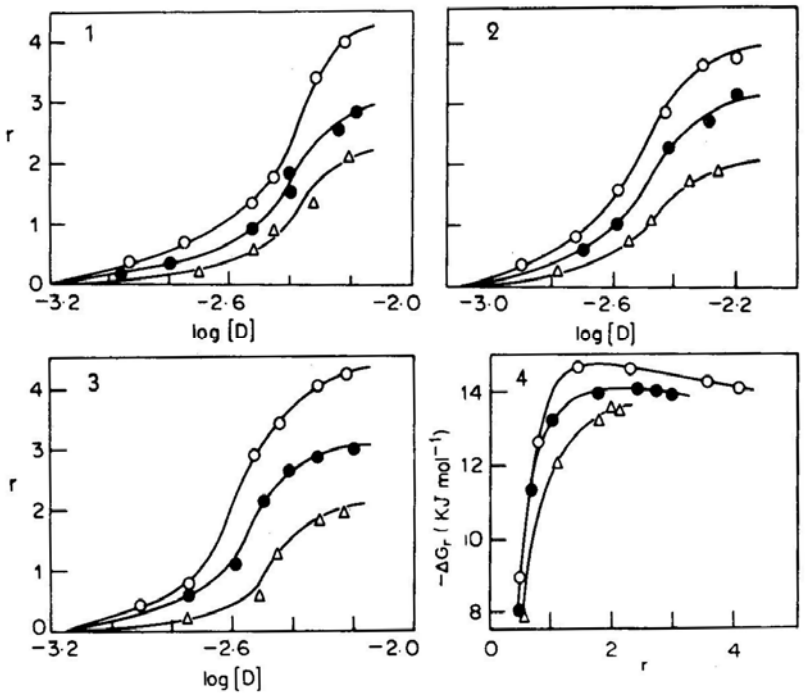
Results and discussion

Binding isotherms obtained for the interaction of lysozyme with penicillin-G, penicillin-V and methicillin showed co-operative binding at pH 5.0,7.0 and 9.0. The binding isotherms of *rvs.* log [D] for these penicillins are shown in figures 1,2 and 3 respectively.

Wyman's binding potential concept was used to interpret these binding isotherms as described by Jones and Manley (1979). The apparent Gibbs free energy of binding, ΔG_a was calculted from *r vs.* log [D] plot from which the apparent free energy of binding per bound ion was calculated using the relation $\Delta G_r = \Delta G_a / r$. The results of the interaction studies with penicillin-G at various pH is shown in figure 4 as a typical example from which the maximum values of ΔG_r are computed. In a similar manner, the ΔG_r values were calculated for penicillin -V and methicillin interactions.

The binding of penicillins with lysozyme decreased slightly with the increase of pH. This was due to the decrease in the net positive charge on the protein with the increase of pH. Accordingly the number of available binding sites on lysozyme decreased from 4 at pH 5.0 to 3 at pH 7.0 and then to 2 at pH 9.0 for all the penicillins. The number of binding sites, the values of ΔG_r and binding constant K at various pH for the interacton of lysozyme with penicillins are given in table 1. It was observed that the magnitude of ΔG_r decreased only slightly with the increase of pH for all penicillins indicating a slight decrease in the strength of binding at higher pH.

The UV difference spectra of lysozyme-penicillin complexes at pH 5.0 showed peaks of low intensity around 284-286 nm in the case of penicillin-G complex, around 296-297 nm in penicillin-V complex and at 300 nm in the case of methicillin complex, thus showing a bathochromic shift in the peaks with the variation in the nature of the side chain group of penicillin nucleus. The intensity of the peaks, however, increased with the increase in the concentration of penicillin in the complex. Figure 5 shows the UV difference spectra of lysozyme-penicillin-G complex taking it as a typical example. None of the penicillin complexes showed any peaks in the 284–300 nm region at pH 7.0 and 9.0 except in the case of lysozyme-penicillin- G complex at pH 7.0 which showed a peak of lesser intensity around 290 nm.



Figures 1-4. 1. Binding isotherms for the interaction of lysozyme with penicillin-G at 25°C: *rvs* log [D]. (O), 0.01 M acetate buffer, pH 5.0;(●), 0.01M phosphate buffer, pH 7.0; (Δ), 0.01 M glycine buffer, pH 9.0. **2.** Binding isotherms for the interaction of lysozyme with penicillin-V at 25° C: *rvs.* log [D]. Notations as in figure 1.**3.** Binding isotherms for the interaction of lysozyme with methicillin at 25° C: *r vs.* log [D], Notations as in figure 1. **4.** Plot of Δ G_r(KJ mol^{-1}) *vs. r* for the interaction of lysozyme with penicillin-G at various pH at 25° C. Notations as in figure 1.

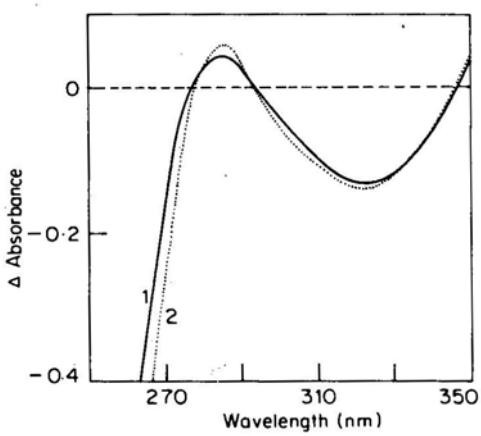


Figure 5. UV difference spectra of 70 μ M lysozyme in acetate buffer, pH 5.0 produced by penicillin-G. Concentration of penicillin-G: 1) 9 mM: 2) 11.25 mM.

Since the penicillins differ only in the nature of their side chains, only penicillin-V and methicillin exhibit intramolecular hydrogen bonding between the side chain and the amide group of penicillin nucleus due to stereochemical considerations. This effect of hydrogen bonding would render these compounds more

Table 1. Interaction of lysozyme with penicillins.

Buffer	pH	Penicillin-G			Penicillin-V			Methicillin		
		Number of binding sites 'n'	$\Delta G_{r,\max}$ (KJ mol^{-1})	K	Number of binding sites 'n'	$\Delta G_{r,\max}$ (KJ mol^{-1})	K	Number of binding sites 'n'	$\Delta G_{r,\max}$ (KJ mol^{-1})	K
Acetate buffer (0.01 M)	5.0	4	−14.760	387.3	4	−15.010	428.5	4	−15.070	439.5
Phosphate buffer (0.01 M)	7.0	3	−14.150	303.2	3	−14.500	349.9	3	−14.800	393.6
Glycine buffer (0.01 M)	9.0	2	−13.670	249.5	2	−14.000	285.8	2	−14.040	290.6

hydrophobic (Bird and Marshall, 1967). Therefore, penicillin-V and methicillin exhibit a higher binding strength compared to penicillin-G as the latter exhibits lower hydrophobicity since no intramolecular hydrogen bonding is possible in it. So the binding strength at any given pH is found to be in the order: penicillin-G<penicillin-V<methicillin. This order is in accordance with our ΔG_r values which are in agreement with those obtained in the case of penicillin-G by other workers (Mitsumori *et al.,* 1980).

The appearance of a peak in the aromatic region of the UV difference spectra of all the penicillin complexes at pH 5.0 and only in the case of penicillin-G complex at pH 7.0 indicates the tryptophan residues as the binding sites involving hydrophobic interactions, in agreement with the observations of earlier workers (Mitsumori *et al.,* 1980). In addition, electrostatic interaction of carboxyl group of penicillins will occur with the cationic residues such as lysines or arginines of the enzyme. That these cationic sites for ionic interactions could be only lysines and not arginines has been shown by Philips *et al.* (1970) from their binding studies of penicillins with lysozyme in which the lysines are modified chemically. Since all the penicillins dissociate into carboxylate anions with pKa values around 2.7 (Rapson and Bird, 1963), these ion binding of penicillins with lysozyme would be occurring at all pH used in this investigation. The non-involvement of tryptophans at pH 9.0 with all penicillins and with penicillin-V and methicillin complexes at pH 7.0, indicates that the binding observed here might be largely due to ionic interactions.

The CD spectra of lysozyme-penicillin complexes at pH 5.0 in the near UV region of 320–250 nm show a slight decrease) in their aromatic positive bands from those of native lysozyme with the increase in the concentration of penicillins in the complex (figure 6A)*. A similar trend was observed at pH 7.0 and 9.0 in this region with all the complexes, but the effect was much less pronounced than at pH 5.0.

In the far UV region of 250–200 nm, the magnitude of the molar ellipticity $[\theta]_{222nm}$ for lyozyme-penicillin complexes at pH 5.0 increased with the increase in concentration of penicillins in the complex, indicating an increase in the α-helical content of native lysozyme (figure 6B)* .The effect of binding on the increase in α-helical content was more with penicillin-G than with penicillin-V and methicillin at pH 5.0. This increase in the α– helical' content generates a more ordered structure since some of the residues supposed to be involved in the interaction lie in the non-helical region of the tertiary structure. Similar trends were observed at pH 7.0 and 9.0 for the complexes of all the penicillins with lysozyme, but only to a lesser degree.

The CD spectra of lysozyme-penicillin complexes in the near UV and far UV regions show that the penicillin-G at pH 5.0 and 7.0 bind at the tryptophan residues, probably Trp-108 lying at the entrance to the cleft of the hydrophobic box and/or Trp-123 lying on the molecular surface involving hydrophobic interactions, and also at the lysine residues, possibly Lys 96, 97 or 116, the former two

* In the CD spectra of penicillin complexes with lysozyme, the effect of binding was more at pH 5.0 than at other pH, and that too with penicillin-G complex. Hence, only the CD spectra of penicillin-G complex in the near UV and far UV regions are given in the figure 6A and B respectively.

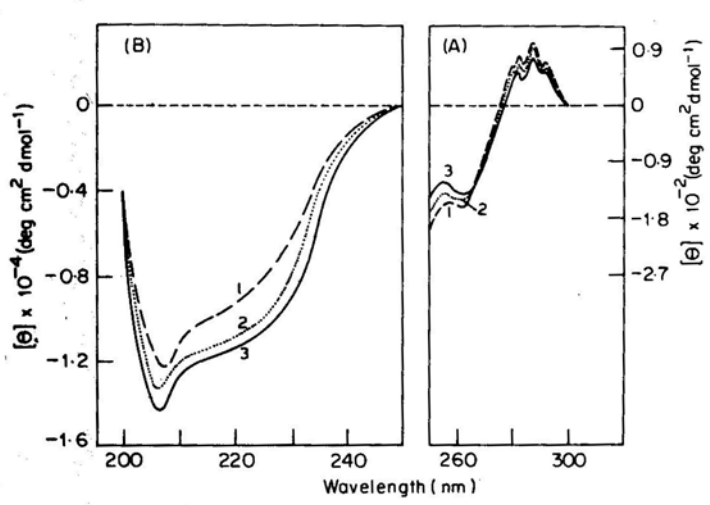


Figure 6. A. Effect of penicillin-G on the CD spectrum of lysozyme in acetate buffer, pH 5.0 (320–250 nm).

Concentration of penicillin-G: 1) 0, M; 2) 0.25 mM; 3) 0.50 mM.

B. Effect of penicillin-G on the CD spectrum of lysozyme in acetate buffer, pH 5.0 (250–200 nm).

Concentration of penicillin-G: 1) 0, M; 2) 0.125 mM; 3) 0.25 mM.

lying at the entrance to the cleft of the hydrophobic box and the latter on the mole cular surface involving electrostatic interactions. But all the penicillins at pH 9.0 and only penicillin-V and methicillin at pH 7.0 bind at these lysine residues involving only ionic interactions.

Acknowledgements

The authors express their sincere thanks to Prof. G. K Narayana Reddy for facilities. They are also highly grateful to Prof. V. Sasisekharan and Prof. K.R. K. Eswaran of the Molecular Biophysics Unit, Indian Institute of Science, Bangalore for the circular dichroism facilities and for their helpful discussion. One of the authors (M.S.) is thankful to the Bangalore University for the award of teacher fellowship.

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