INTERACTION OF LYSOZYME WITH DYES—I: BINDING OF METHYL ORANGE TO LYSOZYME

M. SUBRAMANIAN AND M. P. VENKATAPPA
Department of Chemistry, Central College, Bangalore University, Bangalore 560 001, India.

ABSTRACT

The binding of lysozyme with methyl orange under different conditions of protein concentration and pH ranges has been investigated by equilibrium dialysis technique at 25° C. The linearity of Klotz and Scatchard plots shows simple noncooperative binding involving single type of binding sites. The number of binding sites in lysozyme decreases with the increase of pH. Increase in binding constants with the increase of pH indicates the increased strength of binding at higher pH. However, the selectivity in binding increases with the increased strength of bonding which is evident from the observed decrease in free energy change with the increase of pH.

INTRODUCTION

THE interaction of proteins with ions and neutral molecules is a most useful model for the study of interaction of proteins with dyes, detergents, drugs, antibiotics etc., in biological systems and hence, a means of investigating the conformational changes accompanying such interactions.

Lysozyme, an enzyme with an important biological function, present in hen egg—white is chosen for our studies since its three-dimensional structure in solution and in crystal form has been determined and its physical and chemical characteristics have been investigated extensively[1-4]. It can also be readily isolated and purified in the laboratory. The data on the study of the interaction of lysozyme with dyes are scattered and not comprehensive. The binding studies of lysozyme with 2-*p*-toluidine naphthalene 6-sulphonate at a pH of 4.0 by fluorimetric method[5] have shown that a 1 : 1 complex is formed with a dissociation constant of 0.47, indicating a moderately strong interaction between the dye and the protein. Lysozyme is also found to bind phenol red to a greater extent than other proteins[6]. The binding ability of lysozyme with phenol red is almost constant in the pH range 4 to 10. The binding of lysozyme with Biebrich Scarlet varies with pH; it decreases with an increase in pH from 7.5 to 9. But when the pH is increased from 5 to 7.5, the binding increases surprisingly[7].

In the study of interaction of 1-anilino naphthalene 8-sulphonate by fluorescence titration method[8], the ionic interactions do not play a significant role in the binding. In the case of bromophenol red with lysozyme, lysozyme forms 1 : 1 complex and the dye binds at a site outside the proposed cleft region[9].

In the present communication, the binding of lysozyme with methyl orange at different dye and protein concentrations, and at different pH and ionic strength has been investigated using the equilibrium dialysis technique; the free energies at various pH are also calculated.

MATERIALS AND METHODS

Hen egg-white lysozyme (3× crystallised) from sigma Chemical Co., USA, (lot No. L 6876) and methyl orange (BDH) and reagents of analytical grade were employed in this investigation. Membrane tubing (Union Carbide Corporation, Chicago U.S.A) was used for equilibrium dialysis.

UV-visible spectrophotometer (Carl Zeiss) was used to measure the concentrations of lysozyme and methyl orange solutions. $E^{1\%}_{280\,nm} = 26.4$ was used for the calculation of the concentration of lysozyme[10]. Systronics digital pH meter 335 was employed to measure the pH of the solutions.

In the equilibrium dialysis experiments[11], 2 ml of 0.2% lysozyme in the appropriate buffer solution was taken in the dialysis tubing (10 mm diameter) previously made free of surface active impurities by washing repeatedly with double-distilled water and then with the buffer solution. It was dialysed against 14 ml of methyl orange solutions of varying concentrations in the same buffer medium taken in standard stoppered tubes. The tubes were shaken carefully in a mechanical shaker at 25° C. Controls were carried out under identical conditions using 2 ml of the buffer solution in place of lysozyme solution. After the equilibrium was attained (≈7 hr), the free methyl orange concentration outside the dialysis membrane in both the tubes was measured.

The binding studies have been carried out at varying protein and dye concentrations. The protein concentrations used are 1.4×10^{-4} M and 2.1×10^{-4} M. The complex of lysozyme with methyl orange at pH 5 gets precipitated above the free dye concentration of 5×10^{-4} M, while at pH 7 and 9, there is no such precipitation. Therefore, only in the latter, the binding studies have been carried out between the free dye concentrations 1×10^{-4} M and 8×10^{-4} M.

The binding studies were carried out in acetate buffer at pH 5 with the ionic strengths of 0.00704 and 0.0176, in phosphate buffer at pH 7 with the ionic

strengths of 0.0222 and 0.0555 and in glycine buffer at pH 9 with the ionic strengths of 0.01 and 0.025.

Calculation of Thermodynamic Parameters

In the study of interaction of neutral molecules or ions with proteins, the simplest situation will be the case of multiple binding, involving only one type of binding sites (without interaction between them) on a protein molecule. Then the number of moles (r) of the dye bound per mole of the protein is determined as defined by Klotz[12].

$$r = nk(D)/1 + K(D),$$

where K is the association constant or binding constant, n is the total number of binding sites and (D) is the free dye concentration in equilibrium with the protein in moles per litre. The r values at various concentrations of the dye were calculated as explained earlier[11].

For calculating the thermodynamic parameters Klotz equation of the type

$$\frac{1}{r} = \frac{1}{Kn} \times \frac{1}{(D)} + \frac{1}{n}$$

is used. The equation on rearrangement gives a more convenient form, known as Scatchard equation:

$$\frac{r}{(D)} = Kn - Kr$$

As is evident, the Klotz double reciprocal and the Scatchard plots should be linear in these cases. The binding constants and the free energy changes (ΔG) are calculated from these plots.

Results and Discussion

Figure 1 shows the double reciprocal plot of $1/r$ *vs.* $1/(D)$. The linearity of this plot indicates that only one type of binding sites in lysozyme is involved. The total number of binding sites n on lysozyme and the binding constant K are calculated from this plot. Figure 2 shows the Scatchard plot $r/(D)$ *vs.* r, from which also the values of n and K are calculated. The binding data involving the interaction of methyl orange with lysozyme at various pH values are given in table 1.

At pH 5, the lysozyme molecule has a net positive charge of ≈ 9, while at pH 7 and 9, it has a net positive charge of ≈ 7 and ≈ 6 respectively. The increase in the negatively charged residues with the increase in pH has thus an inhibitory effect on the binding of the

Table 1

Buffer pH	Acetate 5	Phosphate 7	Glycine 9
Binding sites (n)	5	3	2
Binding constant from Klotz plot (K) $\times 10^3$	0.6896	1.111	1.4425
Binding constant from Scatchard plot (K) $\times 10^3$	0.688	1.100	1.44
ΔG (K cals/mole) (± 0.1)	−3.870	−4.153	−4.308

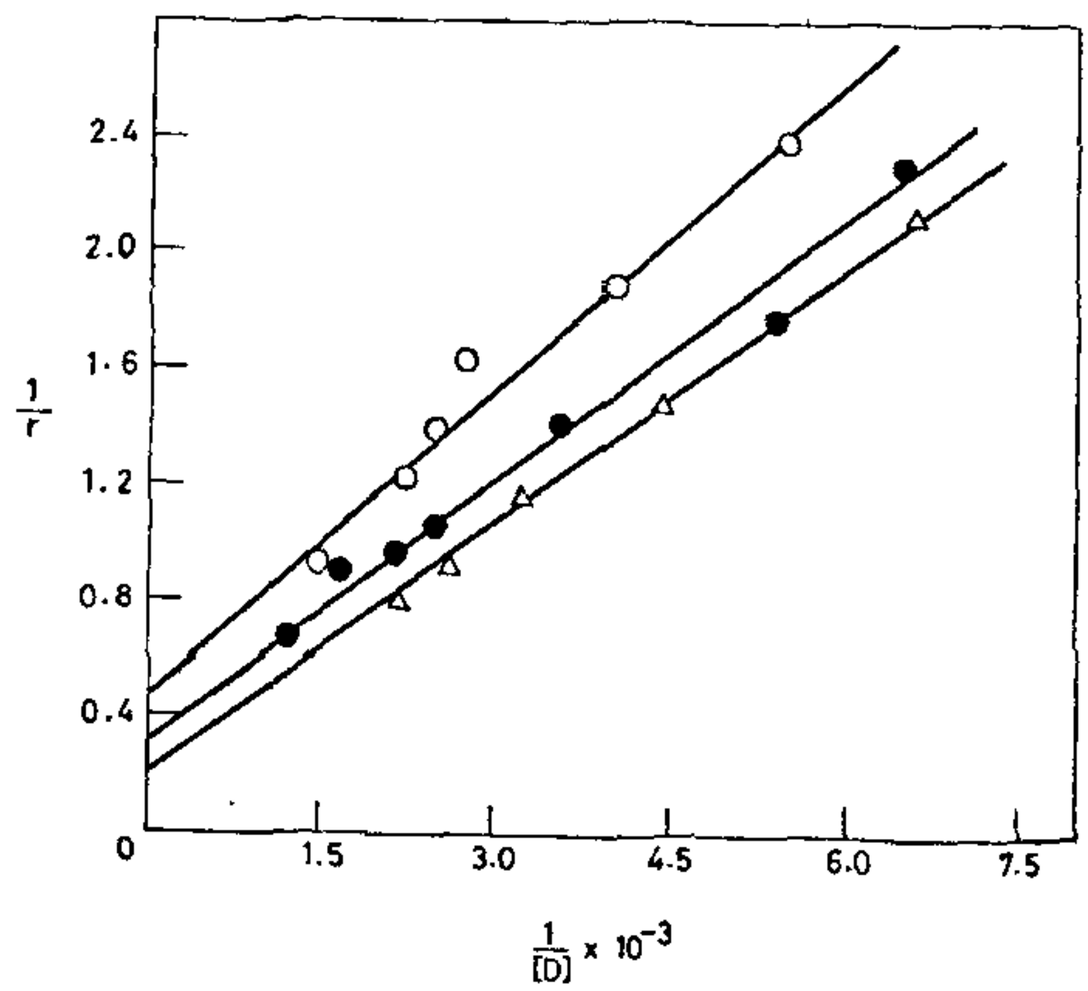

Figure 1. Double reciprocal plot of methyl orange binding with lysozyme. Lysozyme concentration: 1.4×10^{-4} M. Open triangle: acetate buffer (ionic strength 0.00704) pH: 5. Closed circle: phosphate buffer (ionic strength 0.0222) pH: 7. Open circle: glycine buffer (ionic strength 0.01) pH: 9.

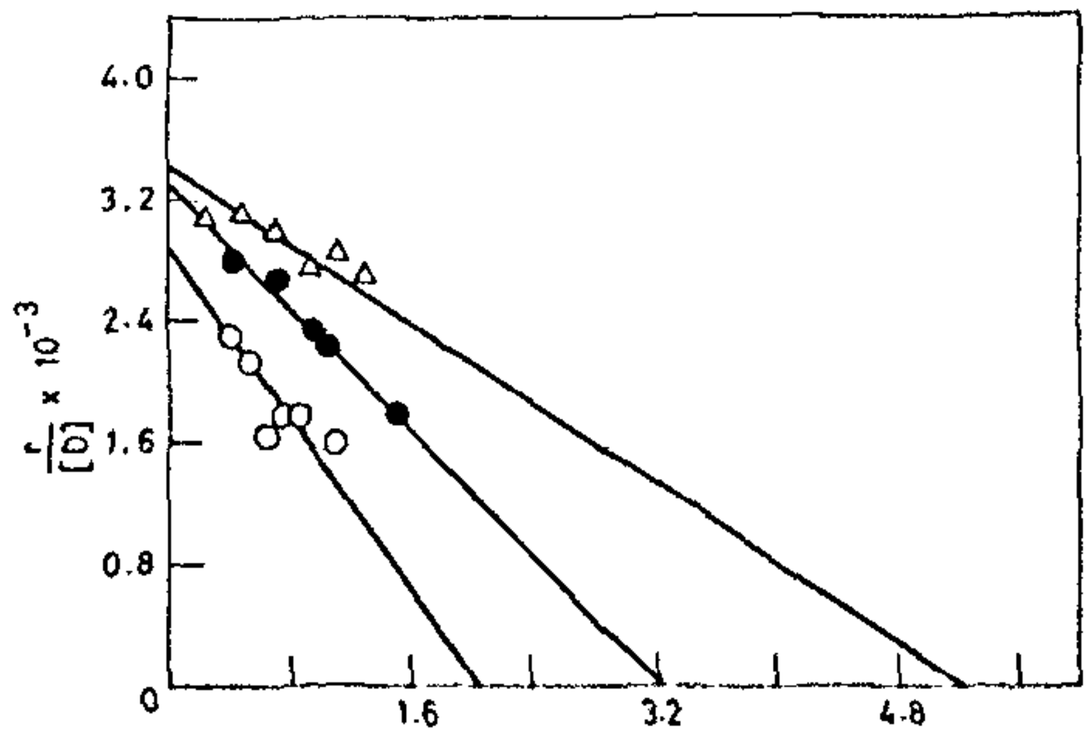

Figure 2. *Scatchard plot for the data shown in figure 1. Details as in figure 1.*

anionic dye. Accordingly, it has been observed that the binding of lysozyme with methyl orange is lowered with the increase of pH. Therefore, the number of binding sites available on the lysozyme molecule has decreased from 5 at pH 5 to 3 at pH 7 and then to 2 at pH 9.

This agrees with the observation of Jones and Manley[13] in their binding studies of *n*-alkyl sulphates with lysozyme in aqueous solutions at pH 3.2 and 9 by equilibrium dialysis method. Increased binding of *n*-alkyl sulphate at pH 3.2 compared with that at pH 9 is due to the increased positive charge on the protein with the decrease of pH.

So, it is probable that the type of binding sites could be lysyl residues or arginyl residues and cannot be both since double reciprocal and Scatchard plots are linear indicating only one type of binding sites involving simple, noncooperative binding. The binding of the dye would be on the surface since all the charged groups are generally distributed on the outside of the tertiary structure of lysozyme.

The values of n, K and $\triangle G$ calculated at various pH, given in table 1, show that the binding constant increases with the increase of pH indicating the increase in the strength of binding at higher pH values. Accordingly, the free energy change ($\triangle G$) decreases with the increase of pH.

The degree of binding at a definite pH falls only slightly with the increase in protein concentration. But it is substantially lowered on increasing the ionic strength of the buffer medium which may be due to the competition for binding between the anions of the buffer medium and those of the dye.

Regarding the kind of bonding involved in this interaction, they could mainly be electrostatic in nature, in addition to the hydrogen bonding and Vander Waals forces.

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