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STUDIES ON SULFAMETHAZINE - LYSOZYME INTERACTIONS BY FLUORESCENCE QUENCHING

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Summary

The binding of sulfamethazine to lysozyme was investigated in relation to certain physicochemical parameters by a fluorescence quenching technique. The parameters considered are ionization of the drug, hydrogen-ion concentration and alteration in protein conformation induced by urea at different pH-levels. The binding constant of the drug was found to be pH-dependent, increasing as the pH is reduced from 9 to 5. On the other hand, denaturation of lysozyme with urea favors the binding process. The interaction being again more pronounced at the lower pH's, when the drug is in the unionized form, and depends also upon the extent of denaturation of the enzyme.

Graphic representation, as well as, computer analysis of the different corrected binding data, at high drug to protein ratios are strongly indica-

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tive of a 1:1 interactions. The nature of the binding forces, and changes in the binding constants associated with alteration in the ionization of sulfamethazine at various pH-points were further interpreted in terms of hydrogen -ion dissociation equilibrium of the drug

Introduction

The binding of ophthalmic drugs to protein constituents of the eye fluids and tissues has been shown on experimental animals to have a tremendous effect on the drugs applied locally to the eye (1). For sulfonamides , it has been found that, only the unbound free drug fraction is active against bacteria (2), susceptible to metabolism (3) and excreted from the kidney (4).

The protein fractions in human tears are mainly albumin, globulins and lysozyme. Although many documents have been accumulated on the binding of drugs to albumin (5,6), and some on the binding to lipoproteins (7), there is scant literature on the binding of drugs to lysozyme (8).

Lysozyme is a low molecular weight cationic protein which tends to associate into dimers and higher polymers depending upon protein concentration, ionic strength, temperature and pH of the medium (9,10). At the concentration, ionic strength and pH of the tears, it is likely to exist as a monomer, and so under the experimental conditions of the current work, lysozyme gives also predominantly monomeric species (11).

The fluorescence of lysozyme arises mainly from its tryptophan residues. Three of the six tryptophan residues are involved in the binding of substrates on lysozyme. The active-site tryptophan residues include tryptophans 62, 63 and 108 (12,13). Studies on the accessibility of residues 62 and 108 to special oxidizing agents revealed that, the bulk of fluorescence of lysozyme is located in tryptophans 62 and 108 (14). The emission from residue 62 is expected to be largest since tryptophan 62 is exposed more completely to the aqueous environment, whereas residue 63 is burried in the interior of the molecule or the non-polar environment (14,15).

In a preceding communication, the binding of a range of ophthalmic drugs to lysozyme was recently investigated by measuring their ability to quench the native fluorescence of lysozyme (16). The present work extends these studies to the interaction of sulfamethazine with lysozyme under the influence of certain criteria commonly encountered in formulation of ophthalmic solutions. The investigated criteria are ionization of the drug, pH of the medium and alteration of the protein conformation by urea at different pH-points. Urea which is known to be an effective denaturant was chosen for such study because it represents also one of the additives commonly used in ophthalmic solutions.

Experimental

Materials and Methods

Hen egg-white lysozyme, 2 times crystallized, lot No. E2-3359 (specific activity 11,710 units/mg), Schwarz/Mann, Orangeburg, New York. Sulfamethazine from Nutritional Biochemical Cor., Clevelan, Ohio. All other chemicals are reagent grade. Deionized water was used throughout.

Lysozyme and drug - lysozyme solutions were prepared in 0.1M phosphate buffer at a fixed protein concentration (1×10^{-4}M); the pH's being adjusted with a Beckman Digital pH-meter model 4500 (Beckman, Fullerton, CA). The molecular weight of lysozyme was taken at 14,400 (17,18). For stabilization of the fluorescence, all lysozyme solutions were aged at least 6 hours before use (19), and then filtered through 0.45 μm pore size Millipore filter (Millipore, Bedford, MA) (20).

The fluorescence measurements were carried out in square quartz cells, 1-cm path length at 22°C, using a Perkin-Elmer MPF-44A spectrofluorimeter (Perkin-Elmer, Norwalk, CT). The excitation wavelength was 305 nm with a slit width of 6 nm and the emission was scanned using a slit width of 7 nm. The emission peak was found near 337 nm for all lysozyme and drug - lysozyme solutions, a finding agree with that reported by Kuramitsu et al (21).

Results and Discussion

The fluorescence spectra of lysozyme and the various drug - lysozyme solutions, at a fixed protein concentration (1×10^{-4}M) and pH 7.4 are shown in Figure 1. It would appear from the figure that, on the addition of the different drug doses, the native fluorescence of the enzyme quenched uniformly and in no instance did the drug emit at any investigated wavelength. The corrected residual fluorescence of 15.5 percent remained at the saturation of lysozyme by the ligand, suggesting that, the fluorescence from tryptophan residues 62 and 108 which are the dominant emitters in native lysozyme (14), are completely quenched.

Figure 2 represents the relative fluorescence intensity plotted as a function of the mole ratio of sulfamethazine to lysozyme. The experimental curve obtained (Fig. 2a) depicts that, at high drug concentrations, the terminal slope of the fluorescence curve is not always horizontal, since there is a significant bulk solution absorption. Correction from this absorption (Fig. 2b) is provided by a procedure originally described by Velick et al (22) and then adopted by Attallah and Lata (19).

Graphic representation, as well as, computer analysis of the different corrected binding data, at high drug to protein ratios are strongly indicative of a 1 : 1 interaction. The binding constants (K) are then determined on the basis of a 1 : 1 interaction by an interative least squares procedure according to the following equations:

$$L + D \rightleftharpoons LD$$

and

$$K = \frac{[LD]}{[L][D]}$$

The concentration of the drug bound [LD] at any given concentration is estimated from the percentage of quenching, allowing the free protein concentration [L] and the free drug concentration [D] to be estimated from the respective total concentrations.

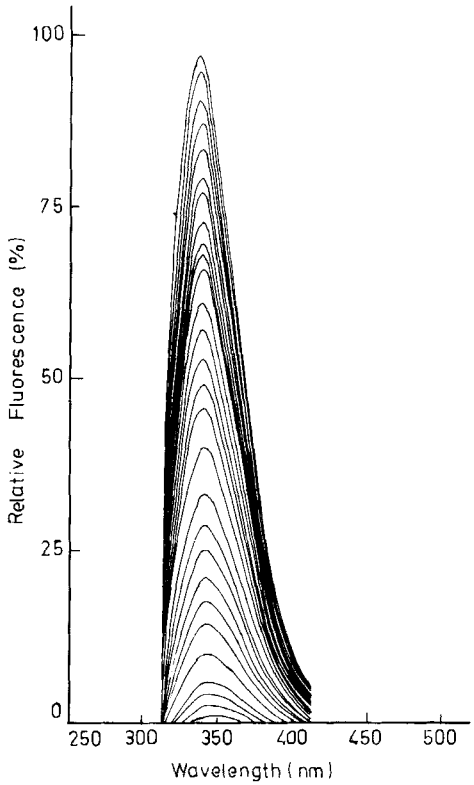

Fig. 1: Fluorescence spectra of lysozyme and sulfamethazine - lysozyme complexes excited at 305 nm. Lysozyme concentration 1 x 10^{-4}M, in 0.1M phosphate buffer and pH 7.4 , at 22°C.

The effect of hydrogen-ion concentration over a pH-range from 5 to 9, on sulfamethazine - lysozyme interactions is shown in Figure 3 and Table 1. It is apparent that, the binding constant (K) of the drug is pH-dependent, increasing as the hydrogen-ion concentration is reduced from 9 to 5. Furthermore, the increase in the binding capacity of the drug, was found to proceed steadily at both pH-extremes, whereas abruptly changed between pH's 7 and 8.5.

In the course of the present work, it was deemed that, anionic drugs like sulfamethazine with a pKa of 7.38 (23), would interact with the cationic lysozyme molecules, and that such interaction increases with increas-

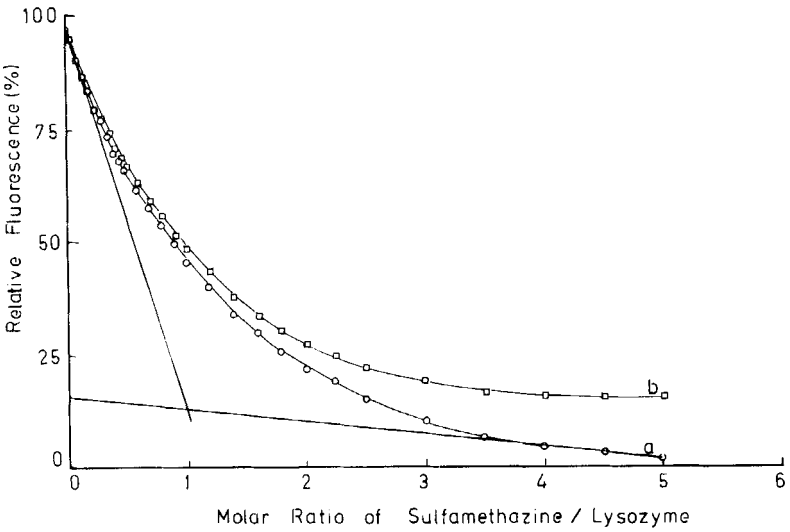

Fig. 2: Relative fluorescent emission at 337 nm as a function of the mole ratio of sulfamethazine to lysozyme. Lysozyme concentration 1×10^{-4} M , in 0.1M phosphate buffer and pH 7.4, at 22°C.

o - Experimental curve (a) and □ - Corrected curve (b)

ing ionization of the drug. However, the experimental findings obtained provide evidence that this is not the case. In contrast, it was found that, the anionic drug species apparently having binding constants to lysozyme of at least an order of magnitude lower than the unionized species, which increase as the pH is shifted to the acid region; a finding indicating that the negative charge is not necessary for such interactions.

Urea denatures lysozyme, the extent of unfolding of the protein depending upon, urea concentration, temperature and pH of the medium (24 - 32). Figure 4 and Table 1 demonstrate the effect of urea over a wide range of concentrations (0 - 8M), on the binding of sulfamethazine to lysozyme, at different pH-levels. At all investigated pH-values, the binding constant of the drug was found to increase linearly with increasing urea concentration; the effect being more pronounced at high urea concentrations (8M), as the protein is denatured. The data obtained reveals that, the binding

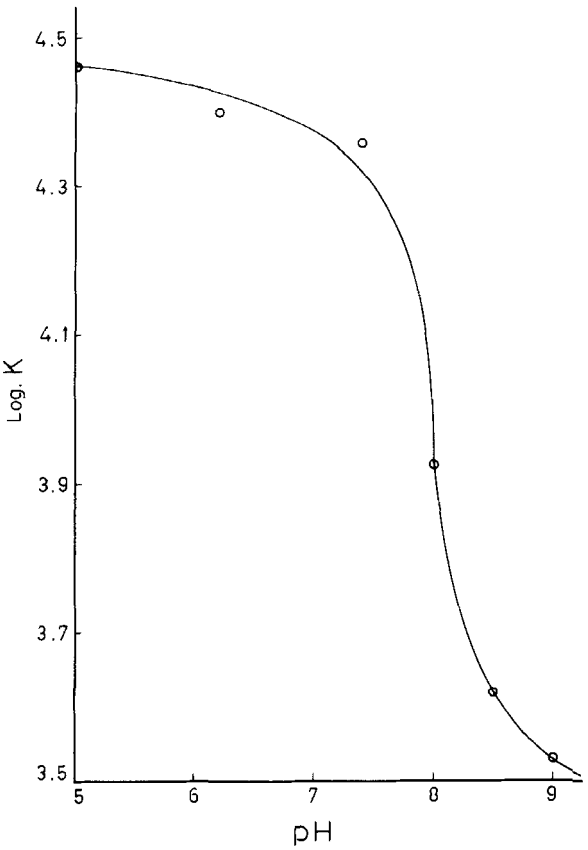

Fig. 3: Effect of pH on the binding constants for sulfamethazine - lysozyme interactions. Lysozyme concentration 1 x 10^{-4}M, in 0.1M phosphate buffer, at 22°C.

capacity is pH-dependent, and again favors at the lower pH's, when the drug is present in the unionized form.

The marked increases in the binding constant of the drug associated with increasing in urea concentration may be attributed to configurational change in the protein structure, and subsequently a more fully exposure of the binding sites, to alteration in the hydration state of lysozyme, to the role displayed by urea on the water structure, which results in a more hydrophobic environment most convenient for the ligand - protein interactions, or a combination of these.

Table 1

Binding Constants for Sulfamethazine - Lysozyme Interactions as a Function of Urea Concentration at Different pH-Levels and 22°C, Assuming a 1 : 1 Interaction.

pH	Urea concentration							
	0		1 M		4 M		8 M	
	K (M^{-1})*	Δ G (Kcal M^{-1})	K (M^{-1})*	Δ G (Kcal M^{-1})	K (M^{-1})*	Δ G (Kcal M^{-1})	K (M^{-1})*	Δ G (Kcal M^{-1})
5.0	29,000 ± 900	-6.02	----------	------	----------	-------	----------	-----
6.2	25,000 ± 800	-5.94	37,000 ± 900	-6.17	57,000 ± 1300	-6.42	79,000 ± 2000	-6.61
7.4	23,000 ± 500	-5.89	31,000 ± 400	-6.06	41,000 ± 900	-6.23	63,000 ± 1500	-6.48
8.0	8,500 ± 200	-5.30	----------	------	----------	-------	----------	-----
8.5	4,200 ± 100	-4.89	6,100 ± 100	-5.11	6,350 ± 100	-5.13	10,000 ± 100	-5.40
9.0	3,400 ± 100	-4.77	----------	------	----------	-----	----------	-----

*All binding constants are expressed at ± 95% asymptotic confidence intervals.

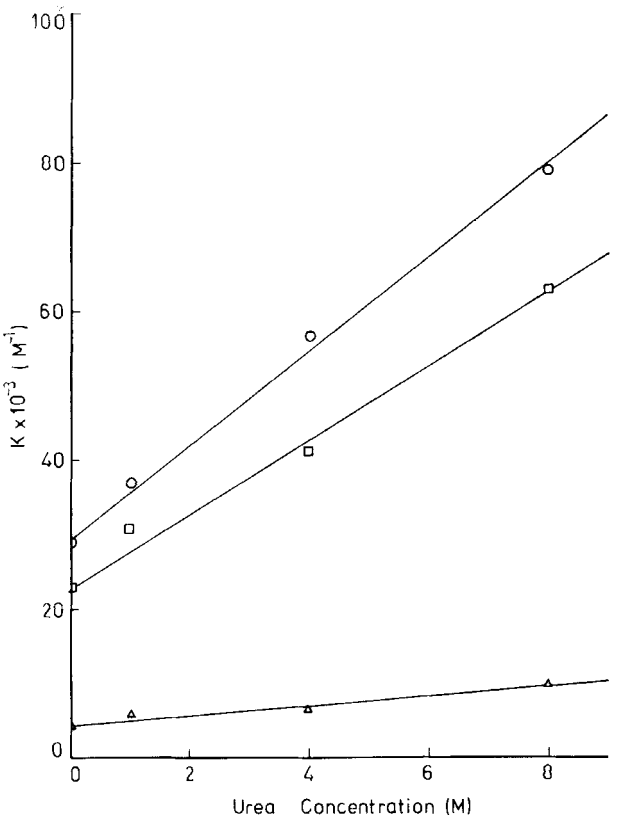

Fig. 4: Effect of urea concentration on the binding constants for sulfamethazine - lysozyme interactions at different pH-levels. Lysozyme concentration 1 x 10^{-4}M, in 0.1M phosphate buffer, at 22°C.

○ - pH 6.2 □ - pH 7.4 △ - pH 8.5

The data obtained presents also some general patterns. Sulfamethazine can bind to a significant extent to lysozyme and this interaction is not primarily due to electrostatic binding forces (33,34), but mainly attributed to hydrophobic phenomena. This finding receives increasing support from the view that, the hydrophobic force is the most form of energy responsible for the binding of sulfonamides to proteins (35 - 37). Furthermore, the data obtained shows a remarkable correspondence with a recent communication by El-Nimr et al (16), on the binding of certain ophthalmic drugs to lysozyme. At pH 7.4 , chloramphenicol a neutral drug

Table 2

Binding Constants for Drug - Lysozyme Interactions at 22°C, Assuming a 1 : 1 Interaction (16)

Drugs	pH	pKa[15]	K (M^{-1})**	Δ G (Kcal M^{-1})
Sulfisoxazole	7.4	5.00	7,000 ± 100	-5.19
Sulfaethidole	7.4	5.60	13,000 ± 200	-5.55
Sulfathiazole	7.4	7.12	21,000 ± 200	-5.83
Sulfamethazine*	7.4	7.38	23,000 ± 500	-5.89
Chloramphenicol	5.0	----	32,000 ± 1000	-6.08
Chloramphenicol	6.2	----	30,000 ± 500	-6.04
Chloramphenicol	7.4	----	30,000 ± 500	-6.04
Chloramphenicol	8.0	----	38,000 ± 1000	-6.18

* Present data

** All binding constants are expressed at ± 95% asymptotic confidence intervals.

was found to exhibit a higher binding constant compared with any other investigated sulfonamide. In addition, the binding constant of chloramphenicol shows no major variations at various pH-levels. Unlike chloramphenicol, the binding of the different sulfonamides to lysozyme was found to be pKa-dependent, where the interaction enhances with reducing ionization of the drug (Table 2).

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