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INVOLVEMENT OF TRYPTOPHAN RESIDUES OF LYSOZYME IN ITS BINDING WITH CYTOSINE ARABINOSIDE*

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Abstract—Cytosine-1-β-D-arabinofuronaside, an antileukemic drug and hen egg white lysozyme (E.C. 3.2.1.17) form a 1 : 1 complex with K_a 1.2×10^4 M^{-1} at low drug concentrations. However, association was cooperative in nature at high concentrations with values of $K_a = 1.8 \times 10^4$ M^{-1} and N = 2. Involvement of tryptophan in the drug protein complex was evident from fluorescence quenching and from the association of the drug with free tryptophan with a $K_a = 1.5 \times 10^4$ M^{-1}. Modification of tryptophan 108 reduced the binding by 89% suggesting a major role for this residue in the binding process. Oxidation of tryptophan 62 and acetylation of lysine residues also decreased the affinity of the drug to the protein by 55 and 66% respectively. Binding improved with increase in temperature and positive values for change in enthalpy and entropy were obtained. Ara-C inhibited lysozyme activity noncompetitively.

A common drug used for treatment of leukemia cytosine arabinoside (Ara-C)† has been shown to inhibit DNA synthesis (S-phase of the cell-cycle) by binding to DNA polymerase III and inhibiting the incorporation of dNTPs [1]. Studies on drug protein interactions are of importance since efficacy of the drug depends on free drug availability. Moreover, this drug is a nucleoside and protein-nucleoside interactions give an insight into protein nucleic acid interactions. Lysozyme levels have been shown to increase some leukaemias and has been used in diagnosis [2]. This protein is also excreted in gram quantities in the urine of these patients [2] and binding of the drug to this protein could even cause excretion of the drug along with protein resulting in an imbalance between bound and unbound drug. Lysozyme has been shown to bind other drugs such as penicillin and tetracycline [3, 4]. In this paper we show that Ara-C binds to lysozyme involving its tryptophan residue(s) and inhibiting its activity noncompetitively.

MATERIALS AND METHODS

Materials. Ara-C, hen egg white lysozyme, *Micrococcus luteus*, cytosine, cytidine and arabinose were all Sigma products. Guanidine hydrochloride was obtained from Schwarz Mann.

Preparation of solutions. A stock solution of Ara-C (1 mg/ml) and hen egg white lysozyme (5 mg/ml) were made in distilled water. The working solution of lysozyme was made by diluting 0.1–0.5 ml of the stock solution to 10 ml with water (pH 4.0, adjusted with dilute hydrochloric acid). The working solutions were made by diluting various aliquots of the stock solution of Ara-C or other ligand solution (10 μl to 0.5 ml) and 0.1–0.5 ml of lysozyme to 10 ml with water (pH 4.0).

All fluorescence spectra were recorded on an Aminco Bowmann Spectrofluorometer with an IP-21 tube and a X–Y recorder. A quartz cell (4 ml) was used. The excitation wave length was 290 nm and emission wavelength was 354 nm.

Calculation of K_a, the association constant. When the Stern Volmer plot of the quenching data yielded a straight line graph the K_a was calculated from the slope of this line assuming 1 : 1 binding [5]. If this plot was non-linear, a Hill plot was drawn in order to yield information about the cooperativity of the binding. The calculations of the thermodynamic constants have also been detailed earlier [5]. Since Ara-C also absorbs at 290 nm a correction for the inner filter effect was carried out according to Velick *et al.* [6].

Modification of lysozyme residues. Trp-62 was oxidized to oxindole using the method of Hayashi *et al.* [7], and Trp 108 was oxidized by iodine according to the method of Imoto *et al.* [8]. Both the modified forms were purified on a chitin coated cellulose column [9]. The NBS-treated enzyme had 50% activity as has been shown by Spande and Witkop [10], while the iodine oxidized enzyme had only 2% of enzymic activity. Both had retained their spectral characteristics, although their fluorescence and absorbance at 280 nm was less than that of the native enzyme. Acetylation with acetic anhydride was carried out according to the method of Neuberger [11]. The extent of acetylation was determined by the Moore and Stein method [12]. Acetylation was found to be complete to the extent of 95%.

Assay of lysozyme activity. Lysozyme activity was measured by monitoring the rate of change of tur-

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† Abbreviations: Ara-C, cytosine-1-β-D-arabinofurnoside; dNTP, deoxynucleotide triphosphate; His, histidine; Trp, tryptophan. NBS, *N*-Bromo succinimide.

bidity of the *M. luteus* cell suspension [13]. The substrate (lyophilized cells) was suspended in 0.06 M phosphate buffer pH 6.2 (1 mg/ml). Buffer (2.7 ml) and the substrate (0.3 ml) were mixed in a cuvette. 10 μl enzyme (10 μg/10μl) was added and stirred well. The optical density at 450 nm was measured on a Hitachi spectrophotometer every 15 sec. The effect of Ara-C on the enzyme activity was measured in phosphate buffer (0.06 M, pH 6.2) containing Ara-C (0.01–0.2 M). Higher concentrations of the drug were used merely for comparison with *N*-acetylglucosamine, the standard inhibitor of lysozyme in which case 0.1 M solutions are normally used.

RESULTS

Ara-C showed no intrinsic fluorescence at 354 nm in the concentration range (3×10^{-5} to 30×10^{-5} M). However, it quenched the fluorescence of lysozyme at 354 nm, without any shift in wavelength as can be seen from Fig. 1. This quenching was observed to be very sensitive to pH by this technique. At a pH higher than 4.5 no quenching was seen suggesting that the interaction at this pH involved protonated forms of both the drug and the protein. Neither ionic strength (0.05–0.2 M) nor ionic species (acetate, phosphate, glycine–sodium hydroxide) influenced the complex formation. The quenching data were modified by the method of Velick *et al.* to correct for the inner filter effect (Fig. 2).

At low concentrations of Ara-C, i.e. up to ten times that of lysozyme a straight line Stern–Volmer graph was obtained (Fig. 3). From this linear graph a $K_a = 1.2 \times 10^4 M^{-1}$ was calculated. When greater concentrations of Ara-C were used, an upward curvature was seen in the Stern–Volmer plot (Fig. 3 inset), and a Scatchard plot of the latter data was also upward sloping. However, the same data yielded a linear Hill plot with a slope N = 2 and an average association constant, $K_a = 1.8 \times 10^4 M^{-1}$ (Fig. 4).

Modification of certain residues of lysozyme had a drastic effect on its binding with Ara-C as seen from Table 1. Oxidation of Trp-108 with iodine reduced the affinity by 88%, whereas NBS oxidation

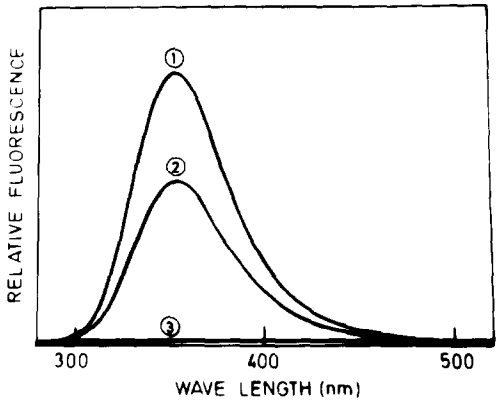

Fig. 1. Quenching of lysozyme fluorescence by Ara-C at pH 4.0. (1) [LZM] = 3.32×10^{-6} M; (2) (1) + (3); (3) [Ara-C] = 35.74×10^{-6} M.

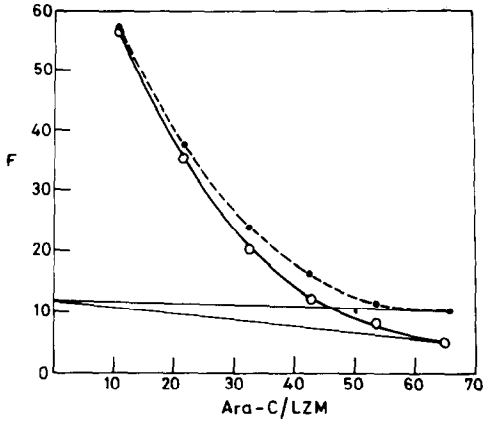

Fig. 2. Correction of fluorescence for the inner filter affect. —— Actual experimental values; ---- corrected quenching of curve. The fluorescence observed, F, is plotted against the mole ratio of Ara-C/LZM.

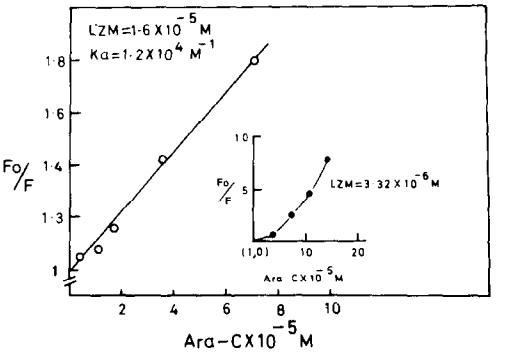

Fig. 3. Stern–Volmer plot for the quenching of lysozyme fluorescence by Ara-C. Fo, fluorescence in absence of Ara-C; F, fluorescence in presence of Ara-C. [Inset], 3.32×10^{-6} M; [lysozyme], 1.66×10^{-6} M.

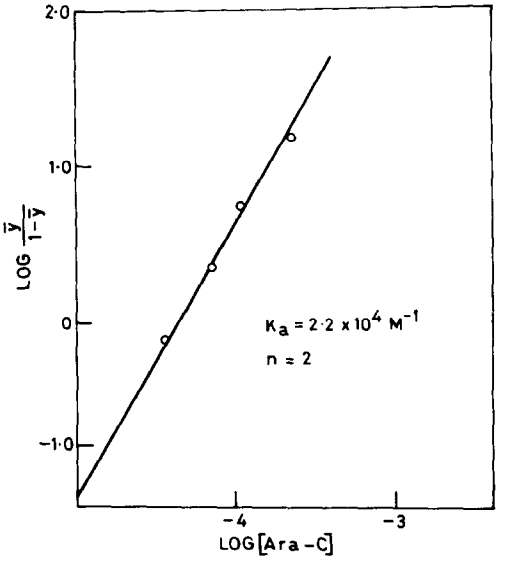

Fig. 4. Hill plot for the association of lysozyme and Ara-C. The data obtained in Fig. 3 (Inset) are used. Y, fractional saturation.

Table 1. Association constants of Ara-C with lysozyme and various modified forms of lysozyme

Proteins	Residues affected	K_a (M^{-1})	Percent reduction in K_a
1. Lysozyme	Nil	1.8×10^4	—
2. Iodine oxidized lysozyme	Trp-108	2.1×10^3	88.5
3. NBS-oxidized lysozyme	Trp-62	8.9×10^3	55.6
4. Acetylated lysozyme	lysine	6×10^3	66.4

The K_a values are an average of four separate experiments.

of Trp-62 caused a 55% decrease in the interaction. Acetylation of all the lysine residues rendered the protein 66% less efficient in binding the drug.

Studies on the effect of temperature on the association constant showed that K_a increased with an increase in temperature. The graphical representation of the Van't Hoff equation depicted in Fig. 5 yielded a slight positive value for $\Delta H = 1.37$ kcals. The change in free energy was calculated to be $\Delta G = -5.8$ kcals at 37° and $\Delta S = 23.1$ esu.

In Fig. 6 is shown the Stern–Volmer plot for the quenching of free tryptophan fluorescence by Ara-C. A linear graph was obtained at concentrations of the drug less than ten times that of the tryptophan, with a $K_a = 1.5 \times 10^4\,M^{-1}$ as in the case of the native protein. At higher concentrations an upward sloping graph was obtained in this case also (Fig. 6, inset). The binding of Ara-C to lysozyme when studied in the presence of 5 M guanidine hydrochloride was found to have a $K_a = 1.7 \times 10^4\,M^{-1}$ (Fig. 7), very close to that observed with free tryptophan and the native enzyme. In this case as well, it was observed that higher concentrations of Ara-C cause a more complex behaviour as indicated by the positive curvature expressed by the Stern–Volmer graph (Fig. 7, inset) at concentrations greater than ten times that of the protein or free amino acid.

The free base cytosine and cytosine riboside also quenched the fluorescence of native lysozyme. In both cases linear Stern–Volmer graphs were obtained. The apparent association constants obtained from these graphs are $K_{a(\text{cytidine})} = 1.5 \times 10^4 M^{-1}$ and $K_{a(\text{cytosine})} = 0.5 \times 10^4 M^{-1}$.

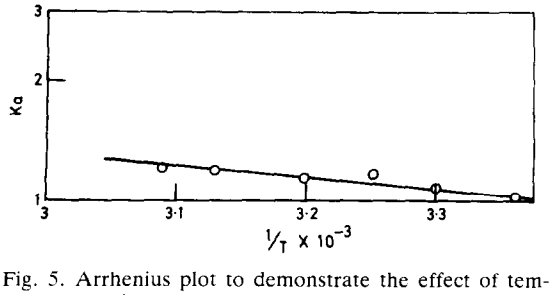

Fig. 5. Arrhenius plot to demonstrate the effect of temperature on lysozyme–Ara-C association. The association constant, K_a, is determined at various temperatures. The concentrations of protein and drug are as in Fig. 3.

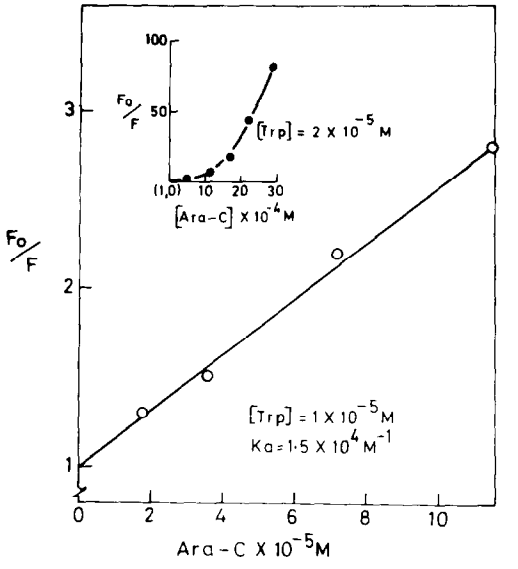

Fig. 6. Stern–Volmer plot for tryptophan–Ara-C association. [Tryptophan] = 1×10^{-5} M. Inset [Tryptophan] = 2×10^{-5} M.

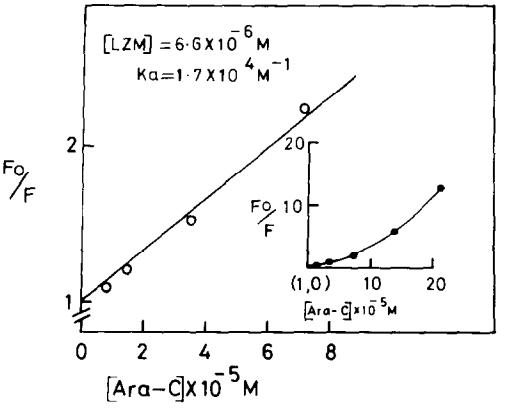

Fig. 7. Stern–Volmer plot for the association of Ara-C and lysozyme in presence of 5 M guanidine hydrochloride. [lysozyme] = 6.6×10^{-6} M.

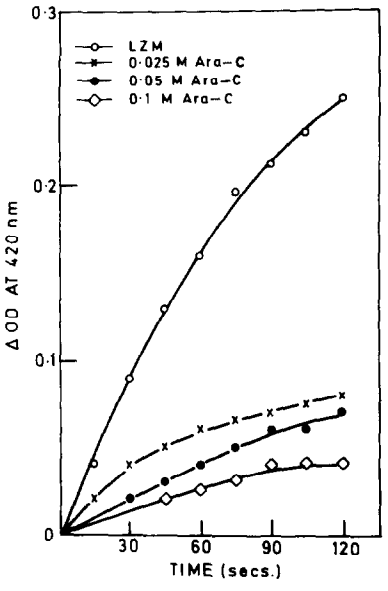

Fig. 8. Effect of Ara-C on the enzymatic activity of lysozyme. The details are in Materials and Methods.

Cytosine has a K_a one fourth that of Ara-C and one third that of cytidine respectively. Arabinose and ribose, both sugars, had no effect on the intrinsic fluorescence of lysozyme.

Ara-C was found to inhibit the enzyme at 0.01 M and above (Fig. 8). 0.025 M Ara-C caused a 74% inhibition. At 0.1 M it seemed to reach maximum efficiency. Kinetics of the inhibition showed a change in both K_m and V_{max}.

DISCUSSION

The quenching of lysozyme fluorescence by Ara-C (Fig. 1) could be due to (a) collisional quenching or (b) due to the formation of a non-fluorescent complex (static quenching) or (c) a complex formation resulting in a change in conformation of the protein and burial of exposed tryptophan residues. The linear Stern–Volmer plot, Fig. 3, could be due to either (a) or (b), as has been shown by Eftnik and Ghiron [14]. We have shown by NMR spectroscopy [15] that Ara-C forms a complex with lysozyme even at 1:1 concentrations. Therefore the quenching of lysozyme by Ara-C is predominantly due to the formation of a non-fluorescent complex. The upward curvature of Stern–Volmer plot at higher concentrations also confirms the formation of a drug protein complex [14]. The Scatchard plot at higher drug concentrations was upward sloping indicating a cooperative phenomenon as described by Mcghee and Von Hippel [16]. This gained further support from the linear Hill plot [17] from which two binding sites on the protein were obtained. Differential Scanning Calorimetry studies carried out by us (unpublished) showed a broadening of temperature transition peak and a significant decrease in the transition temperature of lysozyme in presence of Ara-C (concentration ranging from 1:5 to 1:27, M/M) suggesting that the drug alters the conformation of the protein, corroborating the results obtained from the Hill plot from which a cooperative phenomenon was evident. It therefore appears that at low concentrations of Ara-C, collisonal quenching and static complex formation take place, the latter dominating over the former whereas at higher concentrations of the drug a cooperative phenomenon takes place with a change in conformation of the protein.

The involvement of tryptophan in the Ara-C-lysozyme complex formation was evident from the quenching of the fluorescence of lysozyme arising from its tryptophan residues by the drug. This was further confirmed by the association of Ara-C with the free amino acid, tryptophan. The drug behaved with free tryptophan and denatured lysozyme as it did with native protein Therefore, it can be said that in these cases also both collisional quenching, and quenching due to the formation of a static complex prevail and the latter dominates at high drug concentrations. In these two cases, namely denatured lysozyme and trytophan the question of cooperativity does not arise because one is a free amino acid and the other has all its tryptophan residues exposed.

In order to locate the specific tryptophan residues interacting with Ara-C, Trp-108 and Trp-62 were specifically modified. The binding affinity of Ara-C with lysozyme in which Trp-108 was modified was reduced much more as compared to the one in which Trp-62 was modified. These data indicate that Ara-C first binds to Trp-108 with a higher affinity $1.2 \times 10^4 M^{-1}$ at low drug concentration and then once this site is saturated, binds to Trp-62 at higher concentrations. Since the conformation of these modified enzyme derivatives is not significantly altered [18] it is clear that both these residues are in some way directly involved in the drug-protein association.

Ara-C appeared more efficient in inhibiting the activity of native lysozyme as compared to its well known inhibitor *N*-acetyl glucosamine as is apparent from the lower concentration of Ara-C required to bring about the inhibition. *N*-acetyl glucosamine inhibits lysozyme at concentrations of 0.1 M whereas Ara-C begins to inhibit at 0.01 M concentrations. The noncompetitive inhibition of lysozyme activity by Ara-C goes to prove that the drug binds the enzyme outside its active site region. It has been shown [18] that Trp-108 which is otherwise masked, gets exposed after the enzyme binds its substrate and is involved in the catalytic process of lysing the substrate by helping in distorting the substrate sugar occupying the site 'D' in the active site or cleft region of lysozyme [18]. The fact that Ara-C inhibits the enzyme and that it binds less efficiently to lysozyme in which Trp-108 is modified further supports implication of Trp-108 in the binding process. Acetylation of the lysine residues decreased the binding by 66% showing that a positive environment enhances the binding.

Although cytosine and cytidine bind to the protein they bind in a non-cooperative manner. We have observed that cytidine binds at a site distinct from that of Ara-C by NMR and enzyme activity measurements (to be published elsewhere) whereas ribose and arabinose do not bind to the protein. None of these inhibit the enzyme activity of lysozyme. Arabinose when covalently linked to cytosine, seems

to play an important role in positioning the cytosine in a manner different from that of ribose. It is therefore quite clear that Ara-C and cytidine bind lysozyme differently.

The positive value of ΔH indicates involvement of hydrophobic forces in the complex formation [19]. The negative free energy change suggests a spontaneous entropy driven process supported by the positive value for ΔS. This increase in entropy could be possibly due to the breaking of the water structure around the binding site.

Our earlier NMR data [15] have shown that the His-15 residue of lysozyme is also involved in the binding along with the C-5 and C-6 protons of the pyrimidine ring of Ara-C. The upfield shift in the drug resonances also indicated the involvement of stacking interactions between drug molecules and the aromatic amino acids of lysozyme as suggested by Helene *et al.* [20]. It is reasonable to conclude from the present findings that the drug stacks over the tryptophan residues.

From the studies presented above it is apparent that although Trp-108 is enclosed in a hydrophobic environment [21], it is the primary site of binding for Ara-C, whereas the exposed Trp-62 [21] is the secondary site, indicating that the environment around Trp-108 is more conducive for the binding of Ara-C. That probably explains the involvement of hydrophobic forces as suggested by the thermodynamic data. Therefore at low concentrations of drug, Ara-C binds to Trp-108, whereas at higher concentrations it also binds Trp-62. The positive charges of the amino groups of lysine are also required to maintain the microenvironment for the drug. The quenching does not reach a definite saturation point, showing that the drug molecules possibly stack over one another and induce a conformational change in the protein, exposing some hidden tryptophan residues in the process.

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