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Equilibrium binding studies of the interaction between anthralin and bovine serum albumin

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Summary

The binding of the antipsoriatic drug, anthralin, to calf thymus deoxyribonucleic acid (DNA) and bovine serum albumin (BSA) was examined using fluorescence spectroscopy. Results indicated that anthralin did not bind to DNA but did bind to BSA at physiological pH. Job's method of continuous variation indicated that anthralin formed a 1 : 1 complex with BSA. The investigation employing BSA was conducted at 4 different temperatures, 8.0, 15.4, 25.0 and 34.7 °C. Langmuir's one-site model was directly fitted to the data with an iterative non-linear regression program. The thermodynamic parameters derived from the van't Hoff plot were as follows: $\Delta G = -8.19$ kcal/mol, $\Delta H = -7.85$ kcal/mol, and $\Delta S = +1.12$ e.u. On the basis of experimental evidence it is proposed that anthralin binds to a tryptophan residue on BSA by a hydrogen bonding mechanism.

Introduction

Anthralin (Fig. 1) is an effective antipsoriatic agent which has been used for over 60 years (Shroot et al., 1981; Cavey et al., 1982). The drug's mechanism of action still remains uncertain, although antipsoriatic action via the intercalation of the anthralin molecule between the DNA base pairs has been proposed (Swanbeck and Thyresson, 1965; Swanbeck, 1966; Swanbeck and Lidén, 1966; Swanbeck and Zetterberg, 1971). The ability of anthralin to bind to DNA is, however, controversial (Caron et al., 1982; Melo et al., 1983). Interaction of this drug with other tissue proteins, such as albumin, in the interstitial fluid has also been suggested (Caron et al., 1982; Melo et al., 1983).

The techniques employed thus far to study these binding interactions have primarily been equilibrium dialysis and visible absorption spectroscopy. The rapid decomposition of anthralin at most sets of experimental conditions (Cavey et al., 1982; Melo et al., 1983; Upadrashta and Wurster,

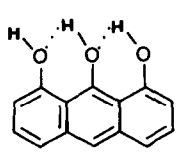

Fig. 1. Structure of anthralin.

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1988a) have made the interpretation of these studies difficult.

In the investigation of the equilibrium adsorption phenomena of labile drugs, it is desirable to make measurements both rapidly and in situ under experimental conditions that are reasonable for the protein involved. The poor intrinsic solubility of this drug necessitates a more sensitive technique than absorption spectroscopy if very small fractions of bound drug are to be measured. Such measurements are necessary in order to completely characterize the binding isotherm.

The aim of this work was to investigate the interaction of anthralin with DNA and BSA in order to definitively establish the presence or absence of binding and to accurately characterize the binding that occurred. The hazards of analyzing binding data with linear transformation methods, such as Scatchard and double-reciprocal plots, are well documented (Klotz, 1974; Deranleau, 1969). Computerized non-linear curve-fitting was thus employed to determine both the number of binding sites per protein molecule and the binding constant for a given binding site. Thermodynamic quantities were also determined so as to gain an understanding of the mechanism of binding.

Materials and Methods

Anthralin was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was kept refrigerated (5 °C) in a desiccator. Its purity was verified using a differential scanning calorimeter (Model DSC-2, Perkin Elmer, Norwalk, CT). Tris(hydroxymethyl)aminomethane (primary standard grade), sodium salt of calf thymus DNA (highly polymerized, lot no. 26F-9610) and bovine serum albumin (fraction V, nitrogen content 15.1%, lot no. 55F-0052) were all obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Certified hydrochloric acid solution (1 N) and potassium chloride (certified A.C.S.) were obtained from Fisher Scientific (Fair Lawn, NJ). Ethanol (95%, U.S.P. grade) was from Midwest Grain Co. of Illinois (Pekin, IL).

Doubly distilled deionized water was used in the preparation of all buffer solutions. Both proteins were dissolved in 0.05 M Tris-HCl (pH 7.4) buffer. The ionic strength of the buffer was maintained at 0.10 M by the addition of an appropriate amount of potassium chloride. The concentrations of DNA and albumin were measured spectrophotometrically with an HP8450A UV-vis spectrophotometer (Hewlett-Packard, Avondale, PA). An $E^{1\%}_{1cm}$ of 6.67 at 280 nm and a molecular weight of 67,000 were used for albumin (Halfman and Nishida, 1972) and a molar absorptivity of 6600 M^{-1} (P) cm^{-1} at 260 nm was used for DNA (Wilson and Lopp, 1979).

Since anthralin had extremely limited solubility in aqueous solutions, a stock solution was prepared in ethanol. The final concentration of ethanol was, however, less than 3.25% in all solutions used in the anthralin–DNA study and less than 1.60% in all solutions used in the anthralin–BSA study.

All pH measurements were made with a Model 611 digital pH meter and a Ag/AgCl glass electrode (Orion Research, Cambridge, MA). The electrode was calibrated at the temperature of the sample using standard buffers of known pH at the same temperature.

Fluorescence spectra were measured with a Shimadzu RF-540 recording spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan). The temperature of the sample, measured in the cuvette, was maintained by circulating constant temperature water through a jacketted cell-holder (Shimadzu Corp., Kyoto, Japan). The protein solutions were kept at the desired temperature using a thermostatted waterbath.

The fluorometric titrations were conducted as follows: 3.0 ml of protein solution of the appropriate concentration was placed in a 1-cm quartz cell and a predetermined volume of anthralin stock solution was added by means of a microliter syringe (Dynatech Precision Sampling Corp., Baton Rouge, LA). The contents were mixed by inversion. Fresh aliquots of the protein and drug solutions were used for each point on the titration curve. For the anthralin–DNA study, DNA solutions (1.82×10^{-6} M) were titrated with aliquots of anthralin stock solution ($\simeq 3 \times 10^{-5}$ M, determined exactly for each titration). A concurrent titration of buffer solution with anthralin

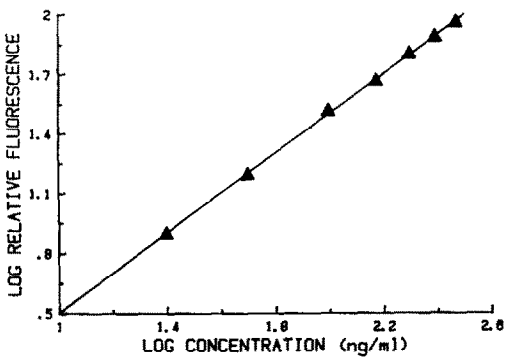

Fig. 2. Calibration plot for anthralin. $\lambda_{excitation} = 387$ nm; $\lambda_{emission} = 518$ nm. The solid line was obtained by linear least-squares regression analysis.

stock solution was performed so that the magnitude of the fluorescence enhancement could be adjusted appropriately. The wavelengths of excitation and emission were 387 and 518 nm, respectively, and both slit widths were 10 nm. For the dilute solutions employed, the calibration plot for anthralin should be linear and this is demonstrated by the data in Fig. 2.

In the studies of the anthralin–BSA interaction, the fluorescence intensity of the native protein in solution (0.58×10^{-6} M) was recorded at excitation and emission wavelengths of 295 and 345 nm, respectively, with both slit widths being 10 nm. The effects of adding various amounts of anthralin stock solution ($\simeq 4 \times 10^{-4}$ M, determined exactly for each titration) on the fluorescence of albumin were monitored. The inner filter effect was minimized by keeping the absorbances of the various solutions at the excitation wavelength less than 0.05 absorbance units. Anthralin itself did not contribute to the observed fluorescence under these conditions.

To determine the maximum number of binding sites per protein molecule, a Job's plot (Job, 1928; Connors, 1987) was constructed. First, the fluorescence intensity of anthralin in the buffer was monitored for solutions having different mole fractions of anthralin. The excitation and emission wavelengths for this purpose were 387 and 518 nm, respectively, with both slit widths being 10 nm. The total fluorescence intensities of several anthralin–BSA solutions, prepared by varying the mole fraction of anthralin and keeping the total concentration of anthralin plus BSA at 2.62×10^{-5} M, were then recorded. The enhancements in fluorescence due to the drug–protein interaction, at different mole fractions of anthralin, were plotted as a function of the mole fraction of anthralin.

Data analysis

The binding equilibria between a small molecule and a protein can be described in terms of: R, the fraction of occupied sites; n_i, the number of binding sites of a given type per protein molecule; and K_i, the intrinsic binding constant for each type of site. When only one type of site is present and the sites are noninteracting, the equation is:

$$R = \frac{nKC_f}{1 + KC_f} \tag{1}$$

where C_f is the concentration of free drug at equilibrium.

For the purpose of determining C_f and C_b, the concentration bound at equilibrium, the fluorescence intensities were expressed as percentages of the initial fluorescence of the protein. C_b and C_f were calculated using Eqns. 2 and 3:

$$C_b = Q_f \cdot [P] \tag{2}$$

$$C_f = C_{total} - C_b \tag{3}$$

where Q_f is the ratio of the quenching at any point on the curve to the maximum quenching, $[P]$ is the total protein concentration and C_{total} is the total concentration of the drug added at any point on the titration curve.

Eqn. 1 (1-site Langmuir model) was fit to the experimentally determined values of R and C_f using an iterative non-linear least-squares regression program for the IBM PC (Enzfitter, Elsevier-Biosoft, 1987). The reported values of n and K were those that yielded the best fit of Eqn. 1 to the data. The values of the association constants (K) were then used in the least-squares linear regression for the van't Hoff plot that was used to determine the thermodynamic parameters.

Results and Discussion

The calibration plot (Fig. 2) of logarithm of relative fluorescence intensity versus logarithm of concentration yielded excellent linearity ($r^2 = 0.9994$) with the slope approaching the theoretical value of unity (0.9993). Any deviation in slope from unity may be diagnostic of side reactions such as decomposition, self-aggregation, and so forth.

If anthralin is immobilized in the presence of DNA by virtue of intercalation, marked changes in the fluorescent properties of the drug should be apparent. However, when solutions of anthralin containing various ratios of DNA/drug (0.01–36) were analyzed, no apparent change in the fluorescence of the drug was evident. The constant fluorescence intensities in the presence and absence of the nucleic acid demonstrated the lack of interaction between anthralin and DNA.

The possibility of external binding should also be addressed. This typically consists of the electrostatic attachment of protonated drug molecules along the negatively charged DNA backbone. However, anthralin exists essentially in the non-ionized form at pH 7.4 and ionic strength equal to 0.1 M ($pK'_a = 9.16$; Upadrashta and Wurster, 1988b) and is negatively charged when ionized. Therefore, electrostatic attraction is not possible and external binding between the drug and DNA is considered unlikely.

The quenching of albumin fluorescence by the binding of drug was accompanied by an increase in the intrinsic fluorescence of the drug. Neither the fluorescence band of albumin nor that of anthralin shifted. It is well established that the intrinsic fluorescence of proteins provides a very sensitive means of studying internal protein structure and changes therein (Chen, 1967). Thus, the change in protein fluorescence was employed in this study to obtain binding data. A typical titration curve for the drug–albumin interaction at 25 °C is shown in Fig. 3. The plateau in the titration curve at high anthralin/BSA mole ratios indicates saturation of the binding site. Since a fresh aliquot of albumin solution and a fresh aliquot of drug solution were used for each point on the titration curve, the photodecomposition of

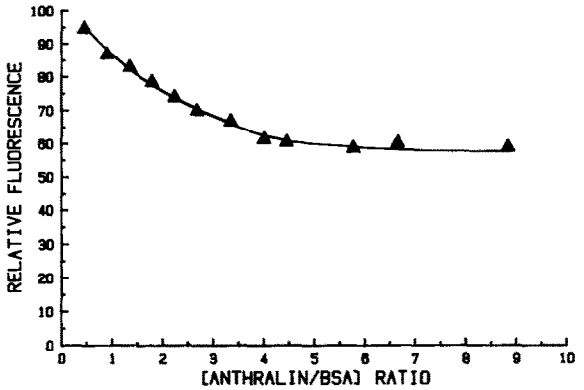

Fig. 3. Fluorescence quenching of bovine serum albumin with increasing anthralin concentration at 25.0 °C and pH 7.4.

the system's components, viz. drug, protein or complex, was minimized. Additionally, the total drug concentration in these experiments was such that even if all the drug had been free, the concentration was too low (Upadrashta and Wurster, 1988b) for self-association to occur, since it was shown that anthralin obeys Beer's law to at least a concentration of 20×10^{-6} M.

In proteins containing tryptophan, the fluorescence spectrum of this residue is observed without interference (Teale, 1960). Bovine serum albumin contains two tryptophans per molecule and thus quenching of tryptophan fluorescence by anthralin provides evidence for the drug's molecular interaction with albumin. This conclusion confirms the results of Melo et al. (1983) who, using visible spectroscopy reported that anthralin formed a complex with human serum albumin.

A Job's plot for determining the stoichiometric ratio of the complex is shown in Fig. 4. The maximum in this plot is at 0.5, the expected value for 1 : 1 complex formation. Thus there is only one binding site for anthralin per protein molecule and it is therefore reasonable to conclude that only one type of binding site contributed to the fluorescence quenching of albumin. Apparently, the microenvironments of the two tryptophans present in each molecule of BSA are different enough that binding only occurs in the vicinity of one of them.

The data from the binding experiments were analyzed by fitting of the appropriate model, i.e.

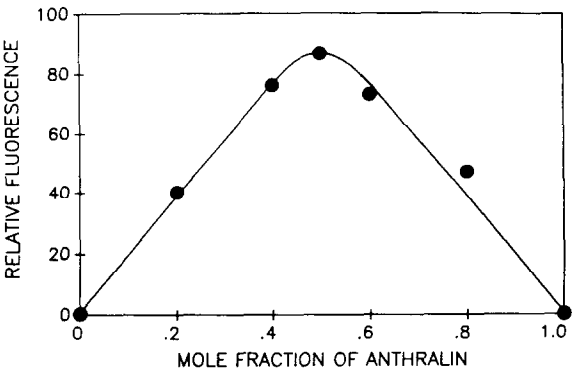

Fig. 4. Job's plot of relative fluorescence intensity for the anthralin–BSA interaction at 25.0 °C and pH 7.4. The total concentration of anthralin and BSA was maintained constant at 26.2 μM.

the 1-site Langmuir model. The binding isotherm (Fig. 5) appears to be type I, according to Brunauer's classification (Brunauer, 1945), and is characterized by a monotonic approach to a limiting adsorption capacity.

The most probable (best-fit) parameters for the anthralin–BSA interaction are summarized in Table 1. Averaging of four replicate analyses yielded: $K = 1.01 \times 10^6$ M^{-1} and $n = 1.28$. It is common to report integral values of n, based on the assumption that n should be an integer with a homogeneous albumin fraction. However, it has long been recognized (Karush, 1950) that bovine serum albumin may not be a single entity with respect to its binding properties. Additionally the albumin concentration was measured spectroscopically using an $E^{1\%}_{1cm}$ of 6.67 at 280 nm. Since the albumin employed (fraction V) in this study is

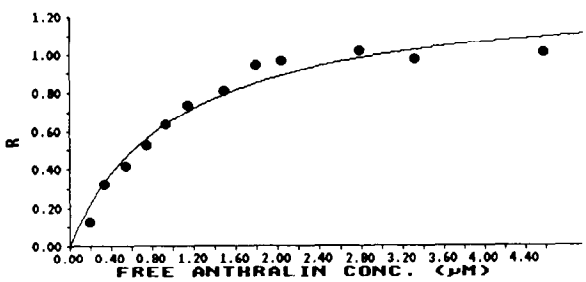

Fig. 5. A typical binding isotherm (single run) for the interaction of anthralin with BSA. Temperature = 25.0 °C; ionic strength = 0.10 M; pH = 7.4. The circles represent the experimental data and the solid curve represents the binding isotherm generated from the computer-fitted model.

TABLE 1

Binding of anthralin to BSA at 25.0 °C, pH = 7.4, and $\mu = 0.10$ M

Run no.	K * E-06	n
1	1.090	1.28
2	0.977	1.28
3	1.000	1.24
4	0.982	1.33
Mean	1.012	1.28
% RSD	5.23	2.83

96–99% pure by label, small alterations in the $E^{1\%}_{1cm}$ value will occur with the consequence of slightly changing the measured protein concentration. Given these possible sources of error, it is clear that the data obtained by monitoring the quenching of the protein fluorescence (with fitting of the Langmuir model to the data) and that obtained by monitoring the increase in anthralin fluorescence (Job's plot) yielded the same stoichiometry. This indicates that the same drug-binding site interaction was being observed in both cases.

In order to elucidate the nature of the binding force between anthralin and albumin, the titrations were also conducted at 3 other temperatures, 8.0, 15.4 and 34.7 °C. Thermodynamic parameters were calculated from the association constants utilizing Eqns. 4–6:

$$\ln K = -\frac{\Delta H}{RT} + \text{constant} \tag{4}$$

$$\Delta G = -\mathrm{RT} \cdot \ln K \tag{5}$$

$$\Delta G = \Delta H - T\Delta S \tag{6}$$

where ΔG is the Gibbs free energy change on binding, ΔH is the enthalpy change for the association of one mole of drug with one mole of the binding sites on albumin, ΔS is the entropy change, and R and T have their customary definitions. The enthalpy change was computed from a van't Hoff plot (Fig. 6) of ln K versus reciprocal temperature according to Eqn. 4. The linearity obtained ($r^2 = 0.9912$) indicated that ΔH was essentially independent of temperature over the range investigated.

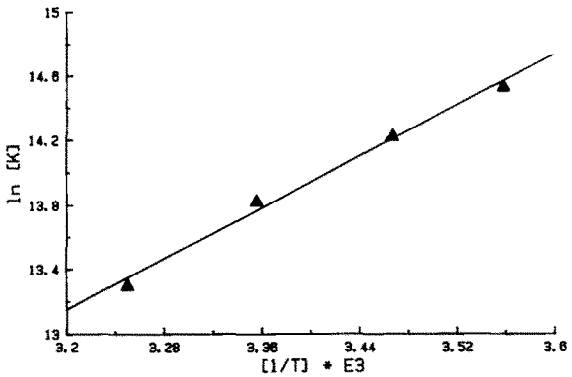

Fig. 6. van't Hoff plot for the binding of anthralin to bovine serum albumin. The solid line was obtained by linear least-squares regression analysis.

The thermodynamic parameters at 25 °C were determined to be: $\Delta G = -8.19$ kcal/mol, $\Delta H = -7.85$ kcal/mol, and $\Delta S = +1.12$ e.u. The decrease in the values of the binding constants with increasing temperature is a direct result of the binding process being exothermic. The value obtained for the enthalpy change upon binding ($\Delta H = -7.85$ kcal/mol) is suggestive of hydrogen bonding between the drug and the binding site. The negative sign for the free energy function, ΔG, indicates, of course, that the binding process is spontaneous. The process of protein unfolding is often significantly endothermic and usually results in large positive changes in the entropy. However, the interaction between anthralin and albumin is exothermic with an insignificant entropy change ($\Delta S = +1.12$ e.u.), which would seem to indicate that the binding site is accessible to anthralin with minimal reorientation of the albumin molecule. A near-zero entropy change would also seem to indicate that hydrophobic forces are not operative.

Since anthralin is a neutral molecule under the experimental conditions, electrostatic forces cannot play a major role in the binding process. Again, the large negative ΔH and near-zero ΔS are in favor of this view since, if electrostatic forces were operative, the value of ΔG should be controlled by ΔS with ΔH having a lesser influence.

In conclusion, the results obtained using an accurate and sensitive fluorescence spectroscopic technique revealed that anthralin did not interact with deoxyribonucleic acid but formed a 1 : 1 complex with bovine serum albumin. The binding constant for this complex was determined to be hydrogen bonding is responsible for the interaction.

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