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Equilibrium binding studies of the 1,8-dihydroxy-9,10-anthraquinone–bovine serum albumin interaction

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Summary

The binding of 1,8-dihydroxy-9,10-anthraquinone (DAQ) to bovine serum albumin (BSA) was investigated using fluorescence spectroscopy. The fluorescence of BSA was quenched following interaction with DAQ and this property was used to generate binding isotherms. Binding studies were conducted at 5 different temperatures: 8.0, 15.4, 25.0, 29.7 and 34.7 °C. The thermodynamic parameters at 25 °C derived from the van't Hoff plot were as follows: $\Delta G = -8.03$ kcal/mol, $\Delta H = -11.8$ kcal/mol, and $\Delta S = -12.6$ e.u. Based on these results it is postulated that DAQ binds to a tryptophan residue on albumin mainly by hydrogen bonding. A statistical technique employing joint confidence ellipsoids has shown that the parameter vectors for anthralin and DAQ are different at the 90% confidence level thus indicating differences in binding affinities. Competitive binding experiments were also conducted and the results indicated that DAQ competitively inhibits the albumin binding of anthralin.

Introduction

Recently it has been shown (Upadrashta and Wurster, 1989) that anthralin, an antipsoriatic agent, binds to bovine serum albumin (BSA). This drug–protein interaction exhibits a 1 : 1 stoichiometry and a binding constant of 1.01×10^6 M^{-1} (Upadrashta and Wurster, 1989). Anthralin rapidly decomposes in aqueous solutions (Upadrashta and Wurster, 1988a, Melo et al., 1983) near neutral pH to yield, principally, 1,8-dihydroxy-9,10-anthraquinone (DAQ, Fig. 1). It has been documented (Elsabbagh et al., 1979) that DAQ is not active against psoriasis. Following topical administration, anthralin is oxidized to DAQ in the skin with DAQ ultimately being excreted in the urine (Ippen, 1981). Although the mechanism of action of

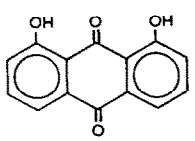

Fig. 1. Structure of 1,8-dihydroxy-9,10-anthraquinone (DAQ).

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topically-applied anthralin in the treatment of psoriasis is unknown (Upadrashta and Wurster, 1989), it can reasonably be assumed that the drug exerts its effects locally and not systemically. Since anthralin would be expected to be highly protein-bound in the interstitial intracutaneous fluid, competition between DAQ and anthralin for protein binding sites would likely have therapeutic consequences. Assuming from structural considerations and some literature precedent (Melo et al., 1983) involving human serum albumin (no binding constant was reported) that DAQ would bind to albumin, studies were initiated to determine the stoichiometry and binding constant for this interaction.

The very low intrinsic solubility of DAQ made it difficult to measure the amount of drug bound at low drug : protein ratios when a typical UV analysis was employed. Such measurements were necessary, however, if the binding isotherm was to be completely characterized and were accomplished by measuring the quenching of the BSA fluorescence upon binding of the DAQ.

Materials and Methods

DAQ and anthralin were obtained from Aldrich Chemical Co. (Milwaukee, WI). The purities of these compounds were verified using differential scanning calorimetry (Model DSC-2, Perkin Elmer, Norwalk, CT). Tris(hydroxymethyl)aminomethane (primary standard grade) and BSA (fraction V, nitrogen content 15.1%, lot no. 55F-0052) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. A fraction V albumin where the impurities (1–4%) were predominantly globular proteins was selected since it was prepared under non-denaturing conditions. Because it was desired to thermodynamically characterize the binding, it seemed that the condition of the albumin was of great importance. Further commercial purification generally requires, amongst other things, multiple temperature fractionation to be employed . Thus it was thought advisable to avoid these materials until their necessity was proven. Certified hydrochloric acid solution (1 N) and potassium chloride (certified A.C.S.) were obtained from Fisher Scientific (Itasca, IL). Ethanol (95%, U.S.P. grade) was from Midwest Grain Co. (Pekin, IL).

Doubly distilled deionized water was used in the preparation of all buffer solutions. BSA was dissolved in 0.05 M Tris-HCl (pH 7.4) buffer. The ionic strength of the buffer was maintained at 0.1 M by the addition of potassium chloride. The concentration of BSA was determined spectrophotometrically with an HP8450A UV-VIS spectrophotometer (Hewlett-Packard, Avondale, PA) assuming an $E_{1cm}^{1\%}$ of 6.67 at 280 nm and a molecular weight of 67,000 (Halfman and Nishida, 1972). Since DAQ had extremely poor solubility in aqueous solutions, a stock solution in ethanol was prepared. The final concentration of ethanol in the measured sample solutions was, however, less than 1.6% in all cases.

All pH measurements were made with a model 611 digital pH meter and a combination glass electrode (Orion Research Incorporated, 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 stock protein solutions were kept at the desired temperature using the same thermostatted waterbath.

The titration procedure consisted of placing 3.0 ml of BSA solution in a 1-cm quartz cell and adding a predetermined volume of DAQ stock solution using 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. The fluorescence intensity of the native BSA solution (0.58×10^{-6} M) was then recorded at excitation and emission wavelengths of 295 nm and 345 nm, respectively, both slit widths being 10 nm. The effects of adding various amounts of DAQ stock solution ($\simeq 4 \times 10^{-4}$ M, determined exactly for each titration) on the fluorescence of BSA were recorded. The

inner filter effect was minimized by keeping the absorbances of the various solutions at the excitation wavelength less than 0.05. DAQ itself did not contribute to the observed fluorescence. The ionization constant of DAQ, under the experimental conditions employed, was determined using a previously described (Upadrashta and Wurster, 1988b) procedure.

Fluorescence spectrophotometry was also employed to examine the effect of DAQ on the binding of anthralin to bovine serum albumin. For this purpose, albumin solutions in the presence of fixed amounts of the competitor, DAQ, were titrated with aliquots of anthralin stock solution according to the procedure described earlier. In all experiments 0.05 M Tris-HCl (pH 7.4) buffer was used. The ionic strength of the buffer was maintained at 0.1 M and the experiments were conducted at 25 ° C. The type of inhibition was identified by means of a Lineweaver-Burk type of analysis (Lineweaver and Burk, 1934; Segel, 1975).

Results and Discussion

Aqueous solutions of DAQ did not fluoresce under the experimental conditions employed. However, the binding of DAQ resulted in the quenching of the BSA fluorescence. This fluorescence quenching was therefore used to obtain the binding data. A typical titration curve for the BSA–DAQ interaction is shown in Fig. 2. The plateau at high DAQ/BSA mole ratios indicates saturation of the binding site. Since a fresh aliquot of BSA solution and a fresh aliquot of DAQ solution were used for each point on the titration curve, the decomposition of the system's components (drug, protein, and complex) was minimized. In proteins containing tryptophan, the fluorescence spectrum of this residue is observed without interference (Teale, 1960). BSA contains two tryptophans per molecule and thus quenching of tryptophan fluorescence by DAQ offers evidence for the drug–albumin interaction.

For the purpose of determining C_f and C_b, the concentrations of free and bound DAQ, respectively, at equilibrium, the fluorescence intensities were expressed as percentages of the initial fluorescence of the protein. C_b and C_f were then calculated according to the following 2 equations:

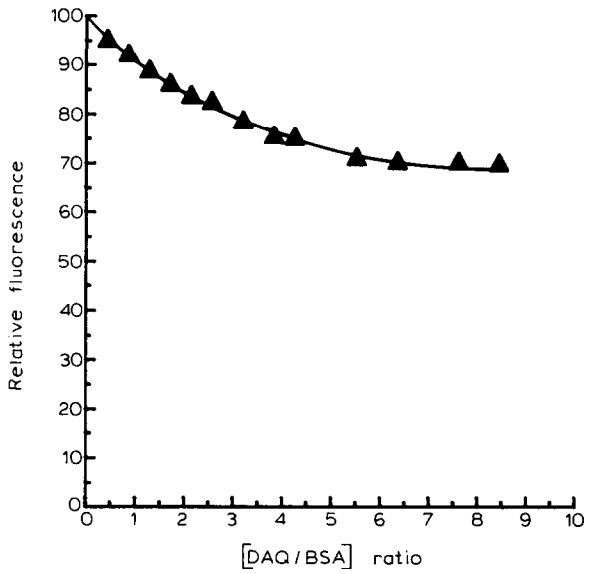

Fig. 2. Fluorescence quenching of BSA with increasing DAQ concentration at 25.0 ° C, pH 7.4 and $\mu = 0.1$ M.

$$C_b = Q_f \cdot [P] \tag{1}$$

$$C_f = C_{total} - C_b \tag{2}$$

where Q_f is the quenching ratio, [P] is the total protein concentration, and C_{total} is the summed concentrations of the free and bound drug. A typical binding isotherm obtained in this manner is shown in Fig. 3.

The equilibrium binding between a ligand 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. If the protein has m classes of independent sites, the first with n_1 equivalent and independent groups, the second with n_2 such groups, and so forth, the general binding model becomes:

$$R = \frac{n_1 \cdot K_1 \cdot C_f}{1 + K_1 \cdot C_f} + \frac{n_2 \cdot K_2 \cdot C_f}{1 + K_2 \cdot C_f} + \cdots + \frac{n_m \cdot K_m \cdot C_f}{1 + K_m \cdot C_f} \tag{3}$$

or

$$R = \sum_{i=1}^{m} \frac{n_i \cdot K_i \cdot C_f}{1 + K_i \cdot C_f} \quad (4)$$

The total number of binding sites, n, is given by:

$$n = \sum_{i=1}^{m} n_i \quad (5)$$

When all potential sites have equivalent affinities and are non-interacting, the relationship between the aforementioned quantities can be represented by the equation:

$$R = \frac{n \cdot K \cdot C_f}{1 + K \cdot C_f} \quad (6)$$

Assuming these conditions hold given the Type I (Brunauer, 1945) binding isotherms obtained, Eqn. 6 was fitted to the experimental values of R and C_f. Best-fit parameter estimates of n and K were obtained using an iterative non-linear least-squares regression program (ENZFITTER, Elsevier-Biosoft, 1987) for the IBM PC. The results of this analysis are summarized in Table 1. The mean of 4 replicate analyses yielded: $K = 0.77 \times 10^6$ M^{-1} and $n = 1.32$ at 25 °C and an ionic strength of 0.1 M. The values for K and n obtained for anthralin binding to BSA under the same experimental conditions (Upadrashta and Wurster, 1989) were 1.01×10^6 M^{-1} and 1.28, respectively. Since in these binding experiments both parameters, n and K, were estimated, there is likely a correlation or covariance between them. Hence, a rigorous comparison of the binding exhibited by the two compounds requires the construction of bivariate confidence regions. Such a procedure was used to compare the parameter vectors of anthralin and DAQ and was accomplished with an interactive BASIC computer program (Munson and Rodbard, 1984). The ln(K) versus ln(n) bivariate 90% joint confidence ellipsoids derived from this analysis are shown in Fig. 4. It is clear that the two ellipsoids representing the respective anthralin and DAQ interactions with BSA do not intersect. This

TABLE 1

Binding of quinone to BSA ($T = 25$ °C, pH = 7.4, and $\mu = 0.1$ M)

Run no.	$K \times 10^{-6}$ (M^{-1})	n
1	0.731	1.38
2	0.774	1.25
3	0.808	1.32
4	0.780	1.34
Mean	0.773	1.32
%RSD	4.10	4.13

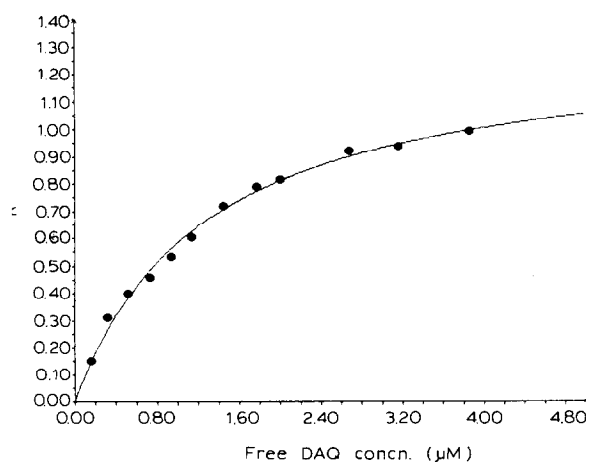

Fig. 3. A typical binding isotherm (single run) for the interaction of DAQ with BSA (25.0 °C; pH = 7.4 and $\mu = 0.1$ M). The circles represent the experimental data and the solid curve represents the binding isotherm generated from the computer-fitted model.

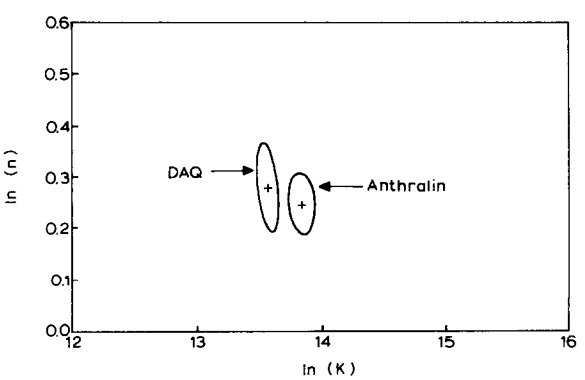

Fig. 4. Joint confidence (90%) ellipsoids for the anthralin–BSA and DAQ–BSA systems.

indicates that the parameter vectors for anthralin and DAQ are different at this probability level and the two compounds are considered to bind to BSA with different affinities.

It can be inferred from the estimated value of n that DAQ only bound to one of the two tryptophan-containing binding sites on BSA. When single-point determinations of the amount of DAQ bound were performed at DAQ : BSA ratios larger than those used for isotherm construction, there was still no evidence for occupation of the second tryptophan-containing site. This apparent difference in the microenvironments of the two tryptophans has been demonstrated previously (Elkana, 1968).

To analyze the binding mechanism(s) involved in the DAQ–BSA interaction, the binding experiments were also conducted at 4 other temperatures: 8.0, 15.4, 29.7 and 34.7 °C. Thermodynamic quantities were calculated using Eqns. 7–9:

$$\ln K = -\frac{\Delta H}{RT} + \text{constant} \quad (7)$$

$$\Delta G = -RT \ln K \quad (8)$$

$$\Delta G = \Delta H - T\Delta S \quad (9)$$

where ΔG is the free energy change on binding, ΔH is the enthalpy change for the binding of one mol of DAQ with one mol of the binding sites, ΔS is the entropy change, R is the gas law constant, and T is the absolute temperature. The quantity ΔH was calculated from a van't Hoff plot (Fig. 5) and the linearity of the plot (correlation coefficient = 0.992) indicated that ΔH was essentially constant over the temperature range investigated.

The calculated thermodynamic parameters for the DAQ–BSA interaction at 25 °C were: $\Delta G = -8.03$ kcal/mol, $\Delta H = -11.8$ kcal/mol, and $\Delta S = -12.6$ e.u. Hydrophobic bonding as described by Kauzmann (1959) and Scheraga (1963) would not result in the negative entropy change observed. The process of protein unfolding with the breaking or straining of bonds results in large positive ΔS values (Tanford, 1980) and is often endothermic. Since DAQ is predominantly non-ionized under the experimental conditions em-

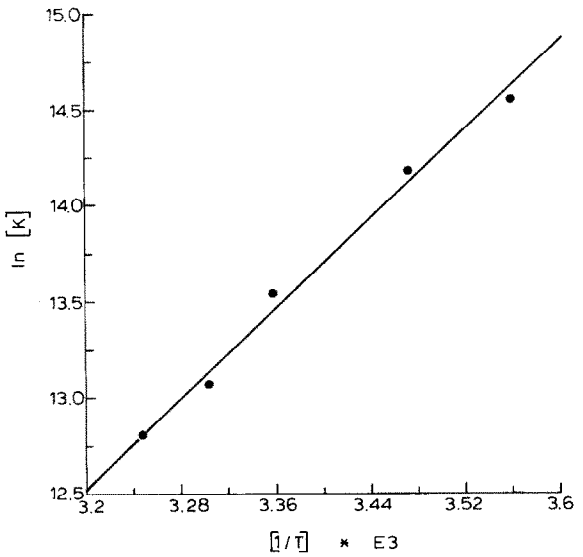

Fig. 5. Van 't Hoff plot for the binding of DAQ to BSA. The solid line was obtained by linear least-squares regression analysis (correlation coefficient = 0.992).

ployed ($pK_a' = 9.29$ at 25 °C and 0.1 M ionic strength) (Upadrashta, 1988), electrostatic forces are unlikely. The sizable negative ΔH value as well as the reasonably large negative ΔS for DAQ support this view since electrostatic bonding should be entropically driven (Klotz, 1973). The magnitude of ΔH (−11.8 kcal/mol) for DAQ is, however, suggestive of strong hydrogen bonding. Based on spectral considerations it has been shown (Ryan and Gibbs, 1970) that testosterone, progesterone, and cortisol perturbed the lone tryptophan residue in HSA and hydrogen bonding of the carbonyl to the binding site was thought to be responsible. The carbonyl group in the 10-position of DAQ can similarly hydrogen bond. This is an attractive hypothesis since either lysine or arginine of the second heptapeptide in Fig. 6 can provide an environment similar to that of the lone tryptophan in HSA and either amino acid could serve as a proton donor for hydrogen bonding. A simi-

– LYS – PHE – TRP – GLY – LYS – TYR – LEU –

– LYS – ALA – TRP – SER – VAL – ALA – ARG –

Fig. 6. Tryptophan residues in BSA.

lar conclusion was reached for the anthralin–BSA interaction (Upadrashta and Wurster, 1989).

The possibility of DAQ inhibiting the albumin binding of anthralin was also investigated. The appropriate equation describing the competitive binding (Klotz et al., 1948) of two drugs is:

$$R = \frac{n \cdot K \cdot C_f}{1 + K \cdot C_f + K' \cdot C_f'} \tag{10}$$

Taking the reciprocals and rearranging yields:

$$\frac{1}{R} = \frac{1 + K' \cdot C_f'}{n \cdot K} \cdot \frac{1}{C_f} + \frac{1}{n} \tag{11}$$

where K' is the binding constant in the presence of the inhibitor, C_f' is the concentration of free inhibitor, and n, K, R and C_f have their usual meaning. In this type of analysis, competitive inhibition results in the same ordinate intercept in a $1/R$ vs $1/C_f$ plot but different slopes. In non-competitive inhibition, the lines of the plot will converge at a point to the left of the ordinate axis (i.e. same abscissa intercept). Again, the slopes are not equal. In the case of uncompetitive inhibition, a series of parallel lines with positive y-intercepts and negative x-intercepts are generated.

Eqn. 11 predicts that a plot of $1/R$ vs $1/C_f$ should yield a straight line for any given free concentration of the inhibitor. Further, it predicts a decrease in the binding of the drug with an increase in the inhibitor concentration. Such a plot showing the effect of DAQ on the albumin binding of anthralin at 25 °C and pH 7.4 is shown in Fig. 7. The straight line obtained with anthralin only and those straight lines obtained for anthralin in the presence of DAQ clearly intersect at the same point on the ordinate axis indicating that DAQ competitively inhibits the binding of anthralin.

The assay employed cannot differentiate between DAQ on the surface and anthralin on the surface since both quench albumin fluorescence. This necessitated the use of reasonably low DAQ (inhibitor) concentrations where the assumption that C_f', and therefore C_b' (the concentration of bound inhibitor), was constant could reasonably be made. To further aid this situation, the range of

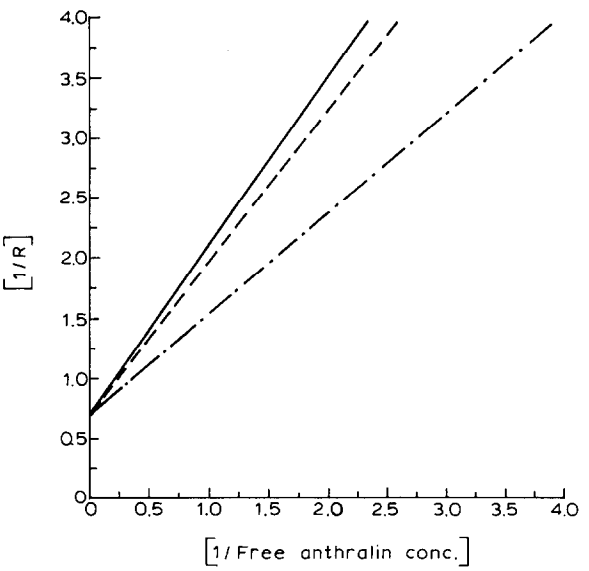

Fig. 7. The effect of DAQ on the binding of anthralin to BSA (25.0 °C, pH 7.4 and $\mu = 0.1$ M). DAQ concentration (μM) and linear least-squares coefficients of correlation are as follows: –·–·–, 0.0, 0.968; — — —, 0.076, 0.992; ———, 0.152, 0.990.

anthralin concentrations employed was decreased from that in the anthralin isotherm determinations (Upadrashta and Wurster, 1989). Since the determination of competitive inhibition by this procedure only requires that the ordinate intercept be independent of inhibitor concentration, it can be concluded that DAQ competitively inhibits the albumin binding of anthralin.

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