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Analysis of Thyroid Hormone Binding to Human Serum Prealbumin by 8-Anilinonaphthalene-1-sulfonate Fluorescence†

Sheue-yann Cheng, Robert A. Pages,‡ Harry A. Saroff, Harold Edelhoch, and Jacob Robbins*

ABSTRACT: Upon binding of 8-anilinonaphthalene-1-sulfonate (ANS) to prealbumin (PA), the absorption maximum is red shifted by 21 nm and the molar extinction coefficient is enhanced by 25%; the fluorescence emission peak of ANS is shifted from 515 to 465 nm with a large increase in quantum yield. At pH 7.4, 2 mol of ANS bind per mol of PA with affinity constants of 3.3×10^5 and 1.0×10^5 M^{-1} as determined by difference absorption spectroscopy and fluorescence enhancement, respectively. Since ANS competes for the thyroxine (T_4) binding sites on PA, the quenching of ANS fluorescence was used to analyze the binding of thyroid hormones and their analogues. Two models were developed to analyze the data. In model I two molecules of T_4 (or analogue) displace two molecules of ANS with quenching only resulting from displacement. By this analysis, the following log K_1 and log K_2 values were obtained: 8.5, 6.7 for T_4; 7.4, 6.1 for 3,5,3′-triiodo-L-thyronine; 7.2, 6.4 for *N*-acetyl-L-thyroxine; 8.2, 6.8 for 4-hydroxy-3,5-diiodobenzaldehyde. These values agree very well with those obtained by equilibrium dialysis. However, in the case of several desamino analogues, model II was employed to analyze the data since it appears that ANS fluorescence is quenched not only by competitive displacement, but also when the analogue occupies the second site. At pH 7.4, the log K_1 values obtained were: 8.2 for 3-(4-hydroxy-3,5-diiodophenyl)propionic acid; 8.4 for 3,5,3′,5′-tetraiodothyroacetic acid; 8.6 for 3,5,3′,5′-tetraiodothyropropionic acid; and 7.7 for 3,5,3′,5′-tetraiodothyrobutyric acid. These values are in accord with those from equilibrium dialysis.

ANS[1] has been widely used to explore the hydrophobic surfaces of proteins (Stryer, 1965; Daniel and Weber, 1966; Edelman and McClure, 1968), as a probe to follow the interactions of an enzyme with a substrate or other cofactors (Brand and Gohlke, 1972; Tu and Hastings, 1975; Seery and Anderson, 1972) and as a label with a long lifetime to measure the rotational behavior of proteins (Weber and Daniel, 1966; Witholt and Brand, 1970). The quenching of ANS fluorescence by competitive displacement has been used to determine the affinities of the thyroid hormones for the single binding site on thyroxine binding globulin (Nilsson and Peterson, 1975a). In the present work with PA, the study of competitive binding has been extended to apply to the case where two molecules of hormones are bound with negative cooperativity.

The structure and number of binding sites of PA have been established recently. X-ray crystallography (Blake et al., 1974) and amino acid sequencing (Kanda et al., 1974) have clearly established that PA consists of four identical subunits. Two identical binding sites for T_4 are located in a central channel formed by a tetrahedral association of the four monomers.

The equilibria for the binding of T_4 have been interpreted recently in terms of two identical sites on PA with negative cooperativity (Ferguson et al., 1975). The binding parameters may also be expressed in terms of two independent sites with different affinities. It was shown by equilibrium dialysis that two molecules of ANS are bound to PA and are competitively displaced by T_4. It was reported earlier by Branch et al. (1971) that the binding of T_4 and its analogues could be followed by the quenching of ANS fluorescence. However, attempts to calculate the binding constants failed since there was no knowledge of the second site for T_4 at that time. With the discovery of the second site (Ferguson et al., 1975), we have

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[1] Abbreviations used: PA, human serum prealbumin; T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; ANS, 8-anilinonaphthalene-1-sulfonate; DIPA, 3-(4-hydroxy-3,5-diiodophenyl)propionic acid; TLC, thin-layer chromatography; Tris, tris(hydroymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

now been able to analyze the fluorescence data in order to obtain the binding parameters. In the case of several desamino analogues of T_4, however, quenching of ANS fluorescence results not only from displacement of the first ANS, but also from quenching of the second ANS on the same molecule. Thus two theoretical models for ANS quenching are required.

Methods and Materials

Human serum PA, obtained from Behring Diagnostics, was purified further by gel electrophoresis as described previously (Cheng et al., 1975). PA concentrations were determined spectrophotometrically at 280 nm ($E_{1cm}^{1\%} = 14.1$) (Raz and Goodman, 1969).

L-T_4, L-T_3, ^{125}I-labeled T_4 and T_3 and their extinction coefficients were the same as described (Pages et al., 1973). ANS-magnesium salt was purchased from Eastman Kodak and three times recrystallized from distilled water; concentrations were determined by absorption at 351 nm ($\epsilon = 6240$) (Ferguson and Cahnmann, 1975). DIPA was prepared by the method of Matsuura and Cahnmann (1959) and concentrations were determined by absorption in 0.01 M NaOH at 310 nm ($\epsilon = 5500$) (Pages et al., 1973). 3,5,3′,5′-Tetraiodothyroacetic acid, 3,5,3′,5′-tetraiodothyropropionic acid, 3,5,-3′,5′-tetraiodothyrobutyric acid, and 3,5,3′-triiodothyropropionic acid were gifts of Warner-Lambert Research Laboratories. The purity of these compounds was confirmed by gas-liquid partition chromatography. The molar extinction coefficient of 3,5,3′,5′-tetraiodothyropropionic acid was determined in 0.01 M NaOH solution and the value of 6100 was found at $\lambda = 325$ nm. The same values were used for the determination of concentration of 3,5,3′,5′-tetraiodothyroacetic acid and 3,5,3′,5′-tetraiodothyrobutyric acid.

Synthesis of 3,5,3′,5′-[^{125}I]Tetraiodothyropropionic Acid. The labeling of this compound was accomplished by iodination of 3,5,3′-triiodothyropropionic acid with carrier-free $^{125}I^-$. Five milligrams of triiodothyropropionic acid in 100 μL of 30% ethylamine was added to a solution of 43 μL of I_2 in ethanol (54 mg/mL) and 2.5 μL of $^{125}I^-$ (100 μCi/μL). The mixture was kept at 0 °C for 15 min. The labeled product was precipitated out by acidifying the resulting solution with 20 μL of 6 N HCl and was extracted with 1 mL of ethyl acetate after addition of 1 drop of saturated aqueous SO_2. The ethyl acetate solution was concentrated to ~5 μL and applied to a silica gel plate (Quantum Industries, Q1-F, 20 × 20 cm) for purification with the solvent system, chloroform–methanol (19:1). The radioactive band corresponding to 3,5,3′,5′-[^{125}I]tetraiodothyropropionic acid was scraped off and eluted with 2 mL of 5% methanol in ethyl acetate. After evaporation of the solvent in a vacuum, the dry residue was dissolved in 10 mM NaOH and used for equilibrium dialysis. The purity was confirmed by thin-layer chromatography followed by autoradiography.

Synthesis of 3-(4-Hydroxy-3,5-[^{125}I]diiodophenyl)propionic Acid. This compound was prepared by exchange labeling with $^{125}I^-$. To a solution of 4.2 mg of DIPA in 200 μL of 80% methanol was added 20 μL of 0.05 M I_2 in absolute methanol. After stirring, 5 μL (0.5 mCi) of carrier-free $^{125}I^-$ was added and the resulting solution was incubated at room temperature for 24 h. After addition of 1 drop of saturated aqueous SO_2 solution and 10 μL of concentrated NH_4OH, [^{125}I]DIPA was purified by passing the resulting mixture through a Sephadex QAE-25 column (column size: 1.5 × 60 cm) equilibrated with 50% methanol. After application, the column was washed with 6 mL of 1% NH_4OH in 50% methanol followed by 10 mL of 50% methanol. Elution of [^{125}I]-DIPA was carried out by batchwise increase of the concentration of acetic acid in 50% methanol solution from 0.033 to 1 M. Fractions were examined by TLC (solvent, chloroform–methanol 19:1) followed by autoradiography. The fractions containing pure [^{125}I]DIPA were pooled, evaporated under vacuum, and used for equilibrium dialysis.

Equilibrium Dialysis. The procedure used was described in detail by Pages et al. (1973).

Fluorescence of ANS. Fluorescence spectra and intensities were obtained with either a Turner 210 or Perkin-Elmer MPF3 spectrofluorometer at 25 ± 1 °C. The excitation and emission wavelengths were 410 and 480 nm, respectively.

Fluorescence titration of ANS with hormones and analogues was carried out by addition of small increments of T_4 or its analogues to 2 mL of a solution of ANS and PA. The concentrations of ANS and PA used routinely were 100–200 and 0.4–1 μM, respectively. The solution was stirred magnetically with a Teflon rod after each addition and the fluorescence recorded. The optical density of the final titration mixture was less than 0.1 at 410 nm.

Polarization of ANS Fluorescence. The Perkin-Elmer MPF3 spectrofluorometer was modified to measure polarization of fluorescence with an automatic polarizing attachment (C. N. Wood Mfg., Newtown, Pa.). The excitation and emission wavelengths were set for 410 and 480 nm, respectively. Polarization is expressed as $(I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$, where I is intensity, $G = I_{HV}/I_{HH}$, and the first and second subscripts refer to the plane of polarization at excitation and emission beams, respectively, where V = vertical and H = horizontal.

Absorption Spectra. Absorption spectra were measured in a Cary 14 spectrophotometer. Difference absorption was obtained by the tandem cell technique.

Buffers. Three buffers were used depending on the pH of the experiments: (a) pH 7.4, 0.05 M potassium phosphate–0.1 M NaCl–1 mM EDTA; (b) pH 8.6, 0.05 M Tris-HCl–0.1 M NaCl–1 mM EDTA; and (c) pH 5.6, 0.1 M potassium acetate–0.1 M NaCl–1 mM EDTA. Stock solutions of T_4 and analogues were prepared in 0.01 M NaOH.

Experimental parameters were evaluated by the method of least squares using nonlinear curve fitting programs employing the steepest descent algorithm or the Marquardt–Levenberg algorithm (Mager, 1972).

Results

Binding of ANS

ANS Absorbance. The absorption spectrum of ANS is red shifted by 21 nm and the molar extinction coefficient enhanced by 25% (at the peaks) when it is bound to PA at a concentration of 147 μM. The change in absorbance between bound and free ANS, i.e., the difference absorbance spectrum, has a peak at 383 nm, and a shoulder at 350 nm with 30% of the absorption at the peak. The difference absorbance data for the binding of ANS to PA are presented in Figure 1. The data are interpreted as the binding of ANS to two equivalent, independent sites on the molecule of PA (Ferguson et al., 1975). Equation 1 may be used to evaluate the binding constant and the molar difference extinction coefficient ($\Delta\epsilon_{383}$) from the difference absorbance, ΔA_{383}

$$\Delta A_{383} = \frac{nK_A c_A}{1 + K_A c_A} \Delta\epsilon_{383} c_{PA} \qquad (1)$$

where n is the apparent number of binding sites, K_A is the binding constant, c_A is the concentration (activity) of the free ANS, $\Delta\epsilon_{383}$ is the molar difference extinction coefficient, c_{PA}

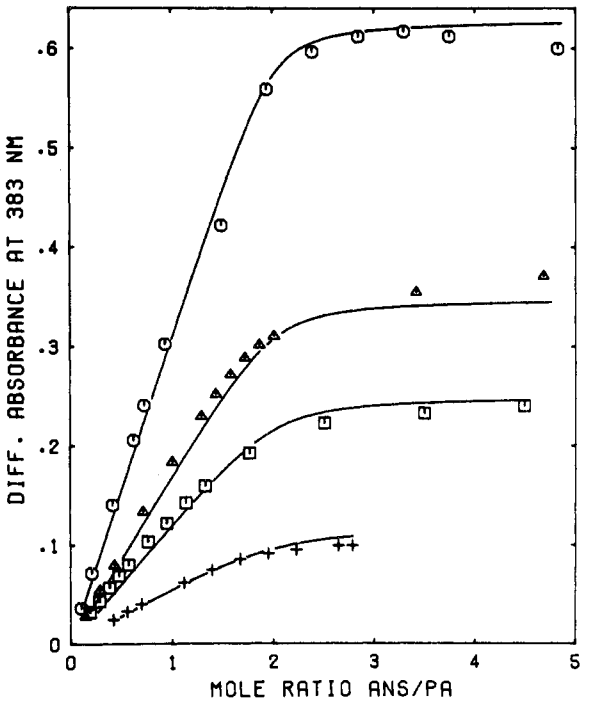

FIGURE 1: Difference absorption titration of four concentrations of PA (174, 96, 69, and 39 μM—going from upper to lower curves) with ANS. The lines are theoretical curves calculated from eq 1 with a binding constant of 3.3×10^5 M^{-1} and 2.0 sites per molecule. The value used for the molar extinction coefficient for ΔA_{383} was 1.96×10^3. Solvent: 0.05 M phosphate-0.1 M NaCl-1 mM EDTA, pH 7.4; $T = 25 \pm 1$ °C.

is the concentration of PA, and $nK_Ac_A/(1 + K_Ac_A)$ is the number of ANS bound to 1 mol of PA. Equation 1 combined with the equation for the conservation of mass for total ANS

$$c_{A(\text{total})} = c_A + \frac{nK_Ac_A}{1 + K_Ac_A} c_{PA}$$

was fit to the data of Figure 1 by the method of least squares to give a value of log $K_A = 5.5$, $n = 2.0$, and a value of $\Delta\epsilon_{383}$ of 1.96×10^3. The four curves of Figure 1 were calculated with these constants.

ANS Fluorescence. The fluorescence of ANS is strongly enhanced and the emission peak is blue-shifted to 465 nm from 515 nm when ANS is bound to PA. Figure 2 shows the binding of ANS at four PA concentrations. As with the absorption data, the fluorescence data were interpreted as the binding of ANS to two equivalent, independent sites. The fluorescence of bound ANS was determined by using a high concentration of PA (binding levels below 1 mol of ANS/mol of PA) at which almost all (98%) of the ANS was bound. Equation 2 was used to evaluate the ANS bound from the relative increase of fluorescence intensity at a given concentration of PA

$$F_{Ai} = \frac{nK_Ac_A}{1 + K_Ac_A} F_Ac_{PA} \quad (2)$$

where F_{Ai} is the fluorescence intensity, and F_A is the molar fluorescence value. Equation 2 combined with the conservation of mass for total ANS was fit to the data of Figure 2 by the method of least squares to give binding parameters at pH 8.6 of log $K_A = 5.0$ and $n = 2.0$. The lines drawn in Figure 2 represent the theoretical curves using these constants.

The parameters of ANS binding to PA at pH 5.6 and 7.4 were also analyzed by ANS fluorescence and the values were obtained by least-squares fit of the data. The log K_A and n values were 5.2 and 1.9 at pH 5.6 and 5.0 and 1.9 at pH 7.4,

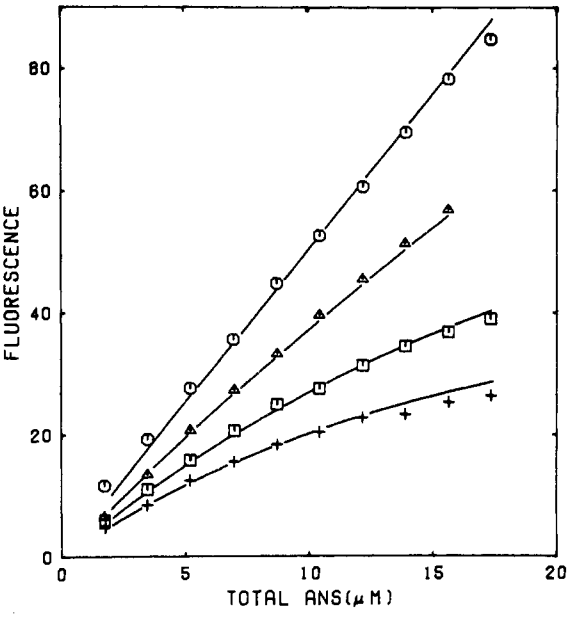

FIGURE 2: Fluorometric titration of four concentrations of PA (158, 15, 7.5, and 4.8 μM—going from upper to lower curves) with ANS. The wavelengths of excitation and emission were 410 and 480 nm, respectively. The lines are theoretical curves for a binding constant of 1.0×10^5 M^{-1} and $n = 2.0$ calculated from eq 2. Solvent: 0.05 M Tris-HCl-0.1 M NaCl-1.0 mM EDTA, pH 8.6; $T = 25 \pm 1$ °C.

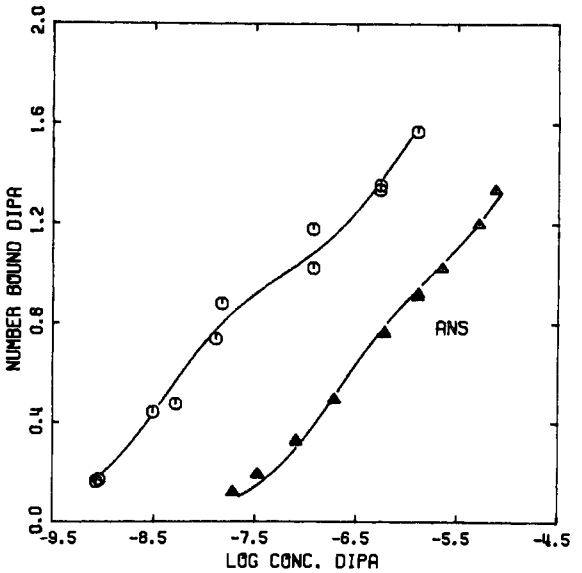

FIGURE 3: Binding of [^{125}I]DIPA to PA (0.89 μM) in the absence (○) and presence (▲) of competitor ANS (0.27 mM) by equilibrium dialysis. The abscissa is the log concentration of free DIPA. The lines are theoretical curves calculated from eq 3 (○) and eq 4 (▲) using constants in Table I. Solvent: 0.05 M phosphate-0.1 M NaCl-1 mM EDTA, pH 7.4; $T = 25 \pm 1$ °C.

respectively. There is evidently very little change of the binding parameters between pH 5.6 and 8.6.

Binding of Analogues by Equilibrium Dialysis

(1) *Absence of ANS.* Figure 3 shows the binding of [^{125}I]-DIPA to PA at pH 7.4 determined by equilibrium dialysis. In accord with an earlier analysis of T_4 binding to PA, the binding constants were computed by the method of least squares using the model with two independent sites represented by eq 3

$$\bar{\nu}_D = \frac{(n/2)K_{D1}c_D}{1 + K_{D1}c_D} + \frac{(n/2)K_{D2}c_D}{1 + K_{D2}c_D} \quad (3)$$

TABLE I: Affinity Constants of Thyroxine and Analogues.

Analogues	pH	Log K_1 ANS fluorescence titration[e]	No.[f]	Log K_1 Equilibrium dialysis	Log K_2 ANS fluorescence titration[e]	No.[f]	Log K_2 Equilibrium dialysis
Model I							
L-Thyroxine	7.4	8.5 (7.9-8.9)	10	8.2	6.7 (6.4-7.1)	10	6.4
3,5,3′-L-Triiodothyronine	7.4	7.4 (7.3-7.5)	2	7.3	6.1 (5.9-6.2)	2	5.9
N-Acetyl-L-thyroxine	7.4	7.2 (7.1-7.3	2	7.1[a]	6.4 (6.3-6.4)	2	6.5[a]
4-Hydroxy-3,5-diiodobenzaldehyde	8.6	8.2	1	8.0[b]	6.8	1	7.0[b]
4-Hydroxy-3,5-diiodobenzaldehyde	7.4	8.6	1	ND[g]	6.7	1	ND
Model II							
3-(4-Hydroxy-3,5-diiodophenyl)-propionic acid	8.6	8.3 (8.3,8.3)	2	8.5			5.3
3-(4-Hydroxy-3,5-diiodophenyl)-propionic acid	7.4	8.2 (8.0-8.5)	4	8.4[c]			6.0[c]
3-(4-Hydroxy-3,5-diiodophenyl)-propionic acid	5.6	ND[g]		8.2			7.3
3-(4-Hydroxy-3,5-diiodophenyl)-propionic acid	7.4	ND[g]		8.6[d]			6.1[d]
3,5,3′,5′-Tetraiodothyroacetic acid	7.4	8.4	1	ND[g]			ND
3,5,3′,5′-Tetraiodothyropropionic acid	7.4	8.6	1	8.4			6.7
3,5,3′,5′-Tetraiodothyrobutyric acid	7.4	7.7	1	ND[g]			ND

[a] From Cheng et al. (1977). [b] Recalculated from the data of Pages et al. (1973). [c] These two constants differ slightly from that reported in the text since $n = 2$ was used for the calculation. [d] Values obtained by competitive equilibrium dialysis in the presence of ANS (0.27 mM). [e] Mean value with range given in parentheses. [f] Number of experiments. [g] ND, not determined.

$$\bar{\nu}_D = \frac{K_{D1}c_D + K_{D2}c_D + K_{D1}c_DK_{A2}c_A + K_{D2}c_DK_{A1}c_A + 2K_{D1}K_{D2}c_D^2}{1 + K_{D1}c_D + K_{D2}c_D + K_{A1}c_A + K_{A2}c_A + K_{A1}K_{A2}c_A^2 + K_{D2}K_{D1}c_D^2 + K_{A1}c_AK_{D2}c_D + K_{D1}c_DK_{A2}c_A} \quad (4)$$

$$\bar{\nu}_A = \frac{K_{A1}c_A + K_{A2}c_A + K_{A1}c_AK_{D2}c_D + K_{A2}c_AK_{D1}c_D + 2K_{A2}K_{A1}c_A^2}{1 + K_{D1}c_D + K_{D2}c_D + K_{A1}c_A + K_{A2}c_A + K_{A1}K_{A2}c_A^2 + K_{D2}K_{D1}c_D^2 + K_{A1}c_AK_{D2}c_D + K_{D1}c_DK_{A2}c_A} \quad (5)$$

where K_{D1} and K_{D2} are the apparent association constants for the binding of DIPA, c_D is the molar concentration of free DIPA (activity coefficient of DIPA is assumed to be 1.0), n is the apparent number of binding sites, and $\bar{\nu}_D$ is the molar ratio of bound DIPA to total PA. At pH 7.4, the least-squares analysis gave log K_{D1} and log K_{D2} values of 8.3 and 5.8, respectively, and $n = 2.1$. Apparent association constants at two other pH values were also determined by the same method and are shown in Table I. With decreasing pH, the difference between the two log K values decreased from 3.2 to 0.9, the change being largely due to variations in log K_{D2}.

Binding of T_3 and tetraiodothyropropionic acid to PA was also determined by equilibrium dialysis at pH 7.4. Analysis of the data by the same procedure gave the binding parameters reported in Table I. Tetraiodothyropropionic acid binds to PA as strongly as DIPA at physiological pH while T_3 binds more weakly.

(2) *Presence of ANS.* Equilibrium dialysis of DIPA in the presence of 0.27 mM ANS was also carried out. The data are presented in Figure 3 as the number of DIPA bound vs. log concentration of DIPA. As expected, the binding curve is displaced to higher DIPA concentrations compared with that observed in the absence of ANS, indicating competition of these two ligands for the same binding sites. The competitive binding of DIPA ($\bar{\nu}_D$) and ANS ($\bar{\nu}_A$) to PA, derived from the two independent sites models (Ferguson et al., 1975), is given by eq 4 and 5, where K_{A1} and K_{A2} are the apparent association constants for the binding of ANS to each site and c_A is the molar concentration of free ANS. Least-squares analysis using eq 4 and 5 gave 8.6 for log K_{D1} and 6.1 for log K_{D2}. These values are in very good agreement with those obtained from equilibrium dialysis in the absence of ANS (Table I). Binding constants of ANS were determined simultaneously in the same experiment. The values obtained were 5.5 and 4.9 for log K_{A1} and log K_{A2}, respectively. These values are in accord with the log K_A values of ANS binding measured either by fluorescence or difference absorption ($K_{A1} = 2K_A$, $K_{A2} = K_A/2$), but are somewhat less than that measured previously by equilibrium dialysis (Ferguson et al., 1975). We have retained these values for the fluorescence procedure of measuring the binding of T_4 and its analogues since they are more consistent with the values obtained directly by fluorescence analysis.

Binding of Analogues by Fluorescence Quenching

Model I. It was shown by equilibrium dialysis that T_4 and ANS compete for the same two sites on PA (Ferguson et al., 1975). Moreover, the fluorescence enhancement resulting from ANS binding to PA can be completely quenched by T_4.

In the absence of T_4, $\bar{\nu}_A$ is determined by eq 3. At each concentration of added T_4, the decrease in $\bar{\nu}_A$ is proportional to the decrease in fluorescence, i.e., $\bar{\nu}_A \propto F$. This is expressed as

$$F_{Ai} = \frac{F_{Ao}\bar{\nu}_{Ai}}{\bar{\nu}_{Ao}} \quad (6)$$

where $\bar{\nu}_{Ao}$ and $\bar{\nu}_{Ai}$ are the number of bound ANS in the absence and presence of T_4, respectively, and F_{Ao} and F_{Ai} are the corresponding fluorescence values. In eq 5 for the competitive binding of T_4 and ANS, since all possible species containing ANS, i.e., $K_{A1}c_A$, $K_{A2}c_A$, $K_{T1}c_TK_{A2}c_A$, $K_{T2}c_TK_{A1}c_A$, and $K_{A1}K_{A2}c_A^2$, contribute to the fluorescence, the relative fluorescence observed should represent the fraction of ANS bound. From eq 4, 5, and 6 in conjunction with the conservation of mass for T_4, $c_{T(total)} = c_{T(free)} + \bar{\nu}_T$PA, the binding constants of T_4 can be calculated.

A typical example of the analysis of T_4 and ANS binding

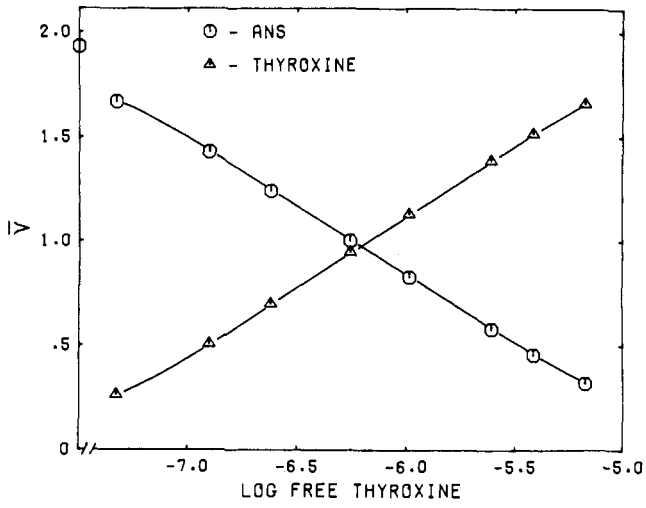

FIGURE 4: Fluorometric titration of ANS-PA with T_4. The concentrations of PA and ANS were 0.88 and 168 μM, respectively. The values of $\bar{\nu}_{ANS}$ were obtained experimentally and those of $\bar{\nu}_{T_4}$ were obtained from eq 4. The theoretical curves from eq 4 and 5 are shown. Solvent: 0.05 M phosphate-0.1 M NaCl-1 mM EDTA, pH 7.4; T = 25 ± 1 °C.

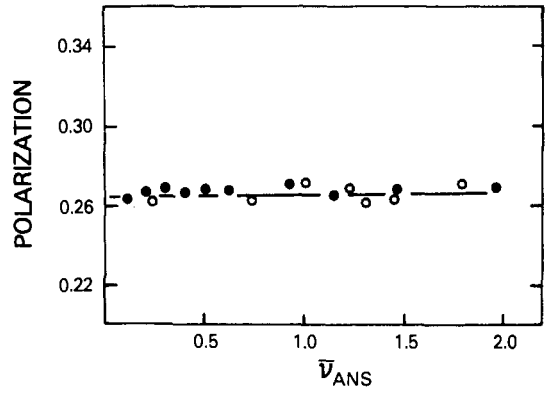

FIGURE 5: Polarization titration by adding ANS to PA, 3.6 μM (○), followed by adding T_4 to ANS-PA (●). The abscissa is the number of bound ANS. Solvent: 0.05 M phosphate-0.1 M NaCl-1 mM EDTA, pH 7.4; T = 25 ± 1 °C.

(by least squares) is shown in Figure 4. When the PA concentration was reduced in half or increased by 50%, the calculated binding constants were the same within experimental error, indicating that they are independent of protein concentration. The log K values of T_4 in ten independent experiments performed over a period of several years were: log K_1 = 8.5 ± 0.5 and log K_2 = 6.7 ± 0.4. The log K values obtained by equilibrium dialysis with [^{125}I]T_4 were 8.2 and 6.4 (Ferguson et al., 1975).

The binding parameters obtained for 3,5,3′-triiodothyronine, N-acetyl-T_4, and 4-hydroxy-3,5-diiodobenzaldehyde by both ANS fluorescence and equilibrium dialysis with labeled compounds are reported in Table I. It is clear that the log K values obtained by fluorescence are in agreement with those obtained by equilibrium dialysis (within ± 0.3 log unit).

Polarization of ANS-PA Fluorescence. Figure 5 shows the polarization of ANS fluorescence with increasing saturation of PA and with decreasing saturation as T_4 displaces ANS. In the absence of T_4, the polarization of ANS was virtually unchanged from 0.2 to 1.8 mol bound. The polarization of ANS remained constant during titration with T_4 as bound ANS decreased from 1.8 to 0.01. Similar results were also found for DIPA. The independence of ANS polarization suggests that the mean lifetime of the excited state of ANS bound to the two

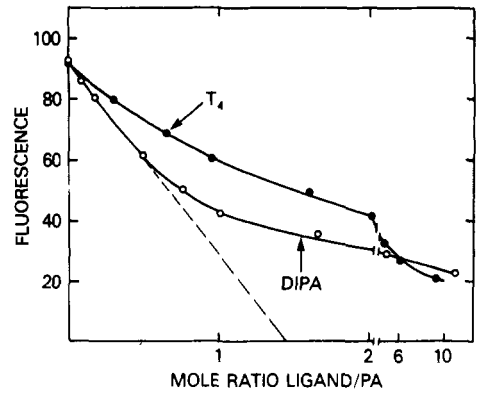

FIGURE 6: Fluorometric titration of ANS-PA with T_4 and DIPA. PA and ANS concentrations were 0.88 and 166 μM, respectively. The lines are theoretical curves calculated from equations in Model I (●) and equations in Model II (○). Solvent: 0.05 M phosphate-0.1 M NaCl-1 mM EDTA, pH 7.4; T = 25 ± 1 °C.

sites in the same. If the second site were modified after the first site was occupied by ANS, a change in polarization would be expected with ANS saturation. The independence of the polarization of ANS with T_4 binding also suggests that T_4 does not affect the properties of the binding site for ANS or the conformation of PA.

Model II. In the course of analyzing the binding of DIPA and certain other analogues by model I, the computed curve did not fit the experimental data and log K_1 did not agree with that obtained by equilibrium dialysis. The log K_1 calculated from model I was three orders of magnitude too large. This can be seen in Figure 6, where the ANS fluorescence curves for T_4 and DIPA binding are quite different, although the log K_1 values by equilibrium dialysis are almost identical (Table I). In comparing the data of DIPA with those of T_4, it is apparent that the first four points on the fluorometric titration curve result in greater quenching of ANS fluorescence than can be obtained with the displacement of one ANS for every DIPA added. This result may be explained by assuming either that one DIPA molecule is displacing one bound ANS and quenching the neighboring one or that it is displacing two ANS molecules. Since the results of equilibrium dialysis were not compatible with the release of 2 mol of ANS after the binding of 1 mol of DIPA, then DIPA must be quenching ANS when bound to the same molecule. When it was assumed that the ANS was not fluorescent when the second site was occupied by DIPA, very good agreement was obtained with the log K determined by equilibrium dialysis (Table I, model II). This treatment allows no fluorescence for the species $K_{D1}c_D K_{A2}c_A$ and $K_{D2}c_D K_{A1}c_A$ of eq 5 and, consequently, eq 6 becomes

$$F_{Ai} = \frac{K_{A1}c_A + K_{A2}c_A + 2K_{A1}K_{A2}c_A^2}{(K_{A1}c_A + K_{A2}c_A + 2K_{A1}K_{A2}c_A^2 + K_{D1}c_D K_{A2}c_A + K_{D2}c_D K_{A1}c_A)} \frac{F_{Ao}}{\bar{\nu}_{Ao}} \bar{\nu}_{Ai} \quad (7)$$

Using eq 7 along with eq 4 and 5 and similar calculations as in model I, the affinity constants of DIPA at two different pH values (8.6 and 7.4) were obtained. The log K_1 values of the homologous series tetraiodothyroacetic acid, tetraiodothyropropionic acid, and tetraiodothyrobutyric acid were obtained as listed in Table I. The log K_1 values are in accord with those obtained from equilibrium dialysis. Log K_2 values can also be computed but these are virtually meaningless since there is only one term left in eq 5 which represents displacement of ANS at site 2 by ligand.

Polarization of ANS fluorescence measurements was in agreement with the assumption in model II since reducing the

binding of ANS from 1.9 to 0.2 by adding DIPA had no effect on the polarization. This indicates that the fluorescence of the ANS bound to the same molecule of PA as DIPA was either unaffected or totally quenched. In this case, it must be totally quenched.

Discussion

ANS competes for the same binding sites on PA as T_4 because of its structural resemblance to T_4 (Cody and Hazel, 1976) and its hydrophobic properties. Interest in the study of the structural requirements for binding of thyroid hormones to PA has prompted us to use fluorescence titration of ANS as a rapid and sensitive method to evaluate the interaction of PA with a number of analogues. However, unlike other studies (Nilsson and Peterson, 1975a; Ketley et al., 1975) which involved only one binding site for the ligand, the hormones and analogues are bound to two identical sites with negative cooperativity.

The spectroscopic characteristics of ANS are modified upon binding to PA in the direction that is observed with ANS itself in going from an aqueous solvent to a less polar medium (Stryer, 1965). The absorption maximum is red shifted and increased in intensity; the fluorescence maximum is blue shifted with a very large enhancement in quantum yield. These spectroscopic changes provide a convenient method of assessing the binding of ANS to PA.

At pH 7.4, ANS difference absorption at 383 nm gave binding parameters of log K_A = 5.5 and n = 2.0 which are similar to those obtained by fluorescence. These values agreed well with those obtained by us by equilibrium dialysis (Ferguson et al., 1975) and by Nilsson and Peterson (1975b) by fluorescence.

PA has been shown by equilibrium dialysis to have two sites for T_4 and is now shown to have this number for five analogues of T_4 (Table I). Despite the difference in structural backbone between T_4 and T_3 (thyronine derivatives) and DIPA (tyrosine derivative), two binding sites were found for both derivatives. At pH 7.4, the log K_1 of T_3 is one log unit less than that of T_4. The binding constants for DIPA are similar to those of T_4, indicating that the second iodine atom on the phenolic ring contributes more to the affinity than the nonphenolic ring of the thyronine derivatives. The negative cooperativity observed in the binding of T_3 and all the analogues studied can be explained by ligand–ligand or site–site interaction as suggested for T_4 binding (Ferguson et al., 1975). It is difficult, however, to distinguish between these two mechanisms with the current data.

One interesting finding in the study of binding of DIPA to PA by equilibrium dialysis at three different pH values is the increase of log K_2 with decrease in pH. The log K_2 values differ by 2 log units between 8.6 and 5.6 while there is only a slight decrease in log K_1. Affinity labeling of PA by N-bromoacetyl-T_4 resulted in the attachment of the carboxymethyl amino group of T_4 to Gly-1, Lys-9, and Lys-15 (Cheng et al., 1977). Since these amino acids are near the entrance to the binding channel (Blake et al., 1974), it is reasonable to assume that the phenolic hydroxyl is deep within the channel. Furthermore, the phenolic hydroxyl group of bound T_4 or T_3 is accessible to solvent since the pK of T_4 bound to PA is ~7.0 (Nilsson and Peterson, 1971). Therefore, a change of pH between 8.6 and 5.6 will change the degree of ionization of the phenolic hydroxyl group of DIPA since the pKs of hydroxyl groups of diiodophenols are between 6 and 7. If the hydroxyl groups of bound DIPA molecules are located near the center of the PA molecule, as postulated from its twofold symmetry, the phenolic groups will interact electrostatically more strongly as they become charged. The decreasing negative cooperativity observed in the binding of DIPA with decreasing pH may be a reflection of electrostatic interactions between the two bound ligands.

Two models have been developed in the analysis of binding of T_4 and analogues by fluorescence titration. Although the mechanisms underlying these two models are very different, their basic assumption is the same, i.e., two molecules of T_4 displace two molecules of ANS per molecule of PA on a one to one basis as evidenced by competitive equilibrium dialysis of T_4 or DIPA in the presence of ANS. The two models differ in that in model I the binding of a ligand has no effect on the fluorescence of ANS located on the second site while in model II the ligand completely quenches the fluorescence of the neighboring ANS. The binding constants obtained by equilibrium dialysis and fluorescence titration were in good agreement for the six analogues studied.

Nilsson and Peterson (1975b) had reported a similar study to obtain the affinity constants of T_4 and T_3 by using a slightly different fluorescence dye, 2-p-toluidinylnaphthalene-6-sulfonate. However, these authors found that PA had 1.2 binding sites for the dye and one major binding site for T_4 or T_3.

It is not clear why DIPA and related analogues completely quench the fluorescence of ANS when it is bound on the same molecule. In a study of ANS binding to tubulin, an allosteric effect has been observed in which the binding of Ca^{2+} or vinblastine enhanced the quantum yield of ANS in an independent site without changing its affinity or number of sites (Bhattacharyya and Wolff, 1975). In the present case, however, polarization studies indicated that there was no change in lifetime of ANS over the entire range of binding with or without DIPA. This definitively eliminated the possibility of a major conformational change of PA induced by DIPA; however, polarization studies cannot exclude subtle conformation changes induced by some of the iodinated amino acids. Until x-ray crystallographic studies with T_4 or DIPA in the binding sites of PA are available, these explanations remain speculative.

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Activation of Histone Gene Transcription in Quiescent WI-38 Cells or Mouse Liver by a Nonhistone Chromosomal Protein Fraction from HeLa S_3 Cells†

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ABSTRACT: Using *Escherichia coli* RNA polymerase, histone genes can be transcribed in vitro from chromatin of S phase HeLa S_3 cells and WI-38 human diploid fibroblasts, but not from chromatin of G_1 phase cells. Histone gene transcription is assayed by hybridization of a ^3H-labeled single-stranded DNA complementary to histone mRNA. Using the technique of chromatin reconstitution, we have recently presented evidence which suggests that this cell-cycle difference in histone gene transcription from HeLa and WI-38 cell chromatin can be accounted for by a component or components of the S phase nonhistone chromosomal proteins which has the ability to render histone genes available for transcription. In the present study we examine the ability of chromosomal proteins from S phase HeLa cells to activate histone gene transcription in chromatin from contact-inhibited WI-38 human diploid fibroblasts or from mouse liver. These results suggest that, when chromatin from either contact-inhibited WI-38 cells or mouse liver is dissociated and then reconstituted in the presence of chromosomal proteins from S phase HeLa cells, the histone genes are rendered transcribable. When the chromosomal proteins from S phase HeLa cells are fractionated with QAE-Sephadex, only one fraction which represents approximately 10% of the total chromosomal protein is found effective in activating histone gene transcription from chromatin of G_1 phase HeLa cells, contact-inhibited WI-38 fibroblasts, or mouse liver. Reconstitution of mouse liver chromatin in the presence of S phase HeLa cell chromosomal proteins does not bring about transcription of mouse globin genes, suggesting that the activation of histone genes does not effect a general activation of gene readout.

In HeLa S_3 cells the synthesis of histones is restricted to the S phase of the cell cycle (Spalding et al., 1966; Borun et al., 1967; Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972), and cell-free translation (Borun et al., 1967; Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972; Pederson and Robbins, 1970; Borun et al., 1975) as well as nucleic acid hybridization data (Stein et al., 1975a) have indicated that histone mRNA is associated with the polysomes only at this time. When chromatin isolated from S phase cells is transcribed in vitro, histone sequences are efficiently transcribed. In contrast, histone sequences are not available for transcription in G_1 chromatin, even though the total amount of RNA transcribed is similar (Stein et al., 1975b). Using the technique of chromatin reconstitution, we have recently presented evidence suggesting that it is the nonhistone chromosomal protein portion of the chromatin that is responsible for this difference in in vitro transcription of the histone genes and that this difference can be accounted for by a component or components of the S phase nonhistone chromosomal proteins having the ability to activate histone gene transcription from G_1 chromatin (Stein et al., 1975c; Park et al., 1976).

Since the expression of histone genes is an integral part of cell proliferation, examining the regulation of histone gene transcription may provide an avenue for understanding the control of the proliferative process. To this end, we have examined the regulation of histone gene transcription not only in continuously dividing HeLa S_3 cells, but also in contact-inhibited WI-38 human diploid fibroblasts which have been stimulated to proliferate (Jansing et al., 1977). When nondividing WI-38 fibroblasts are stimulated to divide, histone

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