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THYROGLOBULIN STRUCTURE-FUNCTION: ANILINONAPHTHALENE SULFONATE BINDING AND THE DEFINITION OF NONPOLAR SITES ON NATIVE BOVINE THYROGLOBULIN

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Summary - Native bovine thyroglobulin has been shown by fluorescence titration and equilibrium dialysis to bind 6 moles of 1,8 anilinonaphthalene sulfonate (ANS) per mole of protein at pH 7.0 in 0.1 M sodium phosphate buffer. The average dissociation constant for this binding is 3 x 10^{-5} M. Analysis of the binding data indicates significant cooperativity in the interaction with the reaction order, J, near 2. The binding of ANS to bovine thyroglobulin is accompanied by a blue shift in the ANS fluorescence maximum of near 50 nm and an increase in fluorescence yield of approximately 60 fold.

Thyroglobulin, the iodinated glycoprotein of the thyroid gland, plays an essential role in the biosynthesis and storage of thyroid hormone (1,2). It serves as a substrate for the iodination and subsequent coupling reactions catalyzed by the peroxidase associated with thyroid membranes (TPO) (3). Because of its large size (M.W. near 660,000) and complex nature, structure-function studies on thyroglobulin have been difficult. Since thyroglobulin must interact with a membrane associated enzyme (TPO), we have investigated the possibility that the protein might contain nonpolar regions which might facilitate its interaction with such membranes.

Anilinonaphthalene sulfonate has been recognized as a polarity probe for nonpolar binding sites on a variety of proteins (4,5,6) due to its environmentally sensitive fluorescence. The binding of ANS to a nonpolar site on a protein is normally associated with a significant increase in fluorescence quantum yield and a blue shift in the fluorescence emission spectrum relative to the unbound probe in solution (5). The data presented in this report, derived from both spectrofluorometric titration and equilibrium dialysis experi-

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ments, describe a heretofore undefined structural feature of the thyroglobulin molecule which may be of significance to the function of this protein.

Materials and Methods

Preparation of Thyroglobulin - Bovine thyroid glands were obtained at a local slaughter house. The thyroids were removed upon sacrifice of the animals and kept on ice until use (within 30 min) or frozen within 10 min in the case of later use. The bovine thyroids were sectioned into 1-2 mm wide slices with a single edged razor, and extracted overnight at 4° in 400 ml of 0.15 M NaCl, pH 7.0 per 100 gm of tissue. When frozen thyroids were used they were partially thawed prior to slicing. All subsequent steps in the purification were carried out at 4° unless otherwise noted. The extract was then filtered through several layers of cheesecloth, made 42% saturated with $(NH_4)_2SO_4$ and stirred for 12 hours. The solution was then centrifuged (12,000 x g) for 20 min. The resulting pellet was dissolved and reprecipitated between ammonium sulfate concentrations of 37% and 44% of saturation as previously described (7). The resulting final pellet from 44% of saturation in ammonium sulfate was resuspended in 0.1% NH_4HCO_3, dialyzed against multiple changes of the same buffer, and lyophilized. The partially purified thyroglobulin was redissolved in 0.1% NH_4HCO_3 and applied to a column of Bio Gel A-15M agarose (Bio Rad Laboratories, Richmond, CA) and eluted with the same buffer. The fractions corresponding to the 19S form of thyroglobulin as judged from analytical ultracentrifugation were pooled and lyophilized. Purified thyroglobulin was stored in this form at -20° until its use in experiments.

Anilinonaphthalene sulfonate was obtained from Eastman Chemicals and recrystallized as the free acid from petroleum ether and then three times from water as the magnesium salt. The distilled water used in all cases was treated with a mixed-bed ion-exchange cartridge and an activated charcoal cartridge prior to a final redistillation in an all glass still.

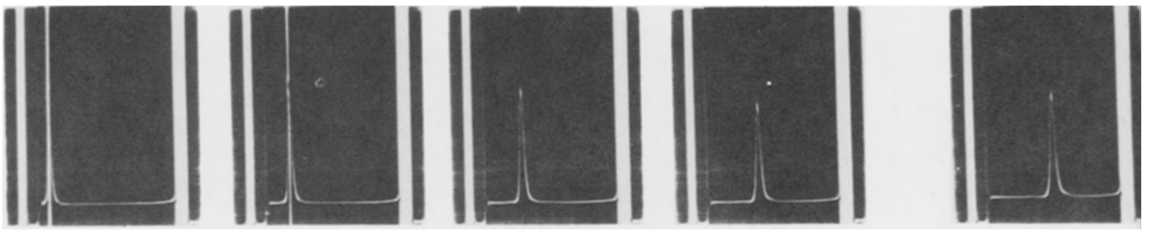
Sedimentation Analysis - Sedimentation velocity analyses were carried out according to Schachman (8) in a Beckman Model E analytical ultracentrifuge equipped with electronic speed control. Schlieren optics were employed with a standard single sector aluminum centerpiece in an AN-D rotor at 48,000 rpm. Photographic plates were read using a Nikon microcomparitor.

Protein Measurements - Protein concentrations were determined from absorbance measurements at 280 nm assuming an $E^{1\%}_{280}$ of 10 for the bovine thyroglobulin and by the method of Hartree (9).

Electrophoresis - Polyacrylamide gel electrophoresis was carried out in 10% acrylamide gel at pH 7.0 in 0.01 M sodium phosphate according to Davis (10) but without a stacking gel. Staining was with Coomassie brilliant blue in methanol:acetic acid:water (227:46:500) and destaining was with a similar mixture of methanol, acetic acid, and water. Gels were scanned at 590 nm in a Gilford spectrophotometer equipped with a gel scanning attachment.

Equilibrium Dialysis - Equilibrium dialysis measurements were carried out in plexiglass chambers which accommodated either 1 or 2 ml samples separated by 6000 MW cut-off dialysis membrane. A solution containing a known concentration of protein in buffer was introduced into the chamber on one side of the membrane and ANS in various amounts was added to an equal volume of buffer alone in the chamber on the opposite side of the membrane. The chambers were sealed and rotated continuously at 2 rpm for 24 hours at 4°. Following equilibration, aliquots were withdrawn from the chambers on each side of the membrane and the absorbance at 366 nm was determined. Bound dye was calculated from the difference in absorbance between the solutions on opposite sides of the membrane. Control experiments with only ANS added to the system confirmed that equilibrium was attained within this time period. The molar extinction coefficient for ANS at 366 nm was assumed to be 4.55×10^3 (11).

Fluorescence Measurements - Fluorescence measurements were obtained initially using an Amicon Bowman SPF spectrofluorometer and later using a Perkin-Elmer MPF 44A spectrofluorometer. Excitation of ANS was at 364 nm (determined



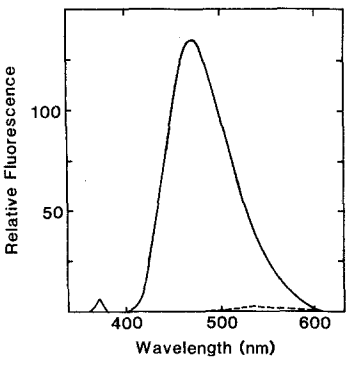
Sedimentation ➔

Figure 1. A Schlieren Photograph Series From A Sedimentation Velocity Run of Purified 19S Bovine Thyroglobulin - Sedimentation was from left to right. Protein concentration was 10 mg/ml in 0.1 M NH_4HCO_3. Photographs were taken at a bar angle of 70° at 8 minute intervals. Rotor speed was 48,000 rpm at a temperature of 20.0°.

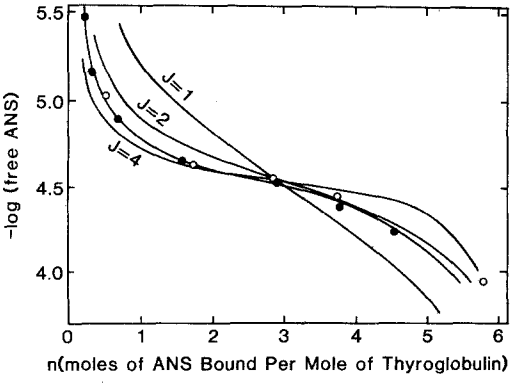
to be an isosbestic point in the absorption spectra of bound and free ANS). Fluorescence titrations were carried out according to Seery and Anderson (11) in 1 cm pathlength cuvettes for solutions of absorbance less than 0.3 and in 2 mm pathlength cuvettes for solutions of higher absorbance. Quinine sulfate titrated into 0.1 M H_2SO_4 was used as a quantum yield reference. Data were treated according to Weber and Young (12), with the fraction of the ANS bound being calculated from the ratio of measured fluorescence to the maximum fluorescence (that fluorescence produced by an identical concentration of ANS when totally bound to thyroglobulin).

Results

The bovine thyroglobulin used in this study was shown to consist of a single major peak in analytical ultracentrifugation with a sedimentation coefficient, $S^{\circ}_{20,w}$, of 18.7 S. The major, 19S, component represented over 90% of the material with the remainder of the material, less than 10%, seen in the form of 12S and 27S species normally found in thyroglobulin preparations (Figure 1). These minor species have been shown to consist of thyroglobulin which is partially dissociated to ½ molecules (12S) or associated to a dimer



(2)



(3)

Figure 2. ANS Fluorescence Emission Spectra in the Present and Absence of Thyroglobulin - Samples contained 1.4 x 10^{-5} M ANS in 0.1 M sodium phosphate buffer, pH 7.0. The dashed line represents the spectrum obtained in the absence of thyroglobulin and the solid line represents the spectrum obtained in the presence of thyroglobulin (20 mg/ml). Excitation was at 364 nm. Both excitation and emission monochromator slits were set at 10 nm band pass.

Figure 3. Titration Data for the ANS-Bovine Thyroglobulin Interaction - A plot according to Bjerrum (14). The solid circles represent data derived from fluorescence polarization. The open circles represent data derived from equilibrium dialysis experiments. The theoretical curves represent binding behavior with Hill coefficients (J) of 1, 2, and 4.

(27S) (13). Polyacrylamide gel electrophoresis of the purified thyroglobulin revealed a pattern identical with known standard of bovine thyroglobulin (15).

Addition of bovine thyroglobulin to a solution of ANS in 0.1 M sodium phosphate buffer at pH 7.0 resulted in a 60-fold increase in the observed fluorescence intensity and a blue shift in the emission maximum of approximately 50 nm (Figure 2).

This interaction between bovine thyroglobulin and ANS was further characterized in fluorescence titration experiments. The data are summarized in the form of a Bjerrum plot (14) in Figure 3). These data indicate an average K_D of 3.0 x 10^{-5} M with an apparent stoichiometry of 6 ANS molecules bound

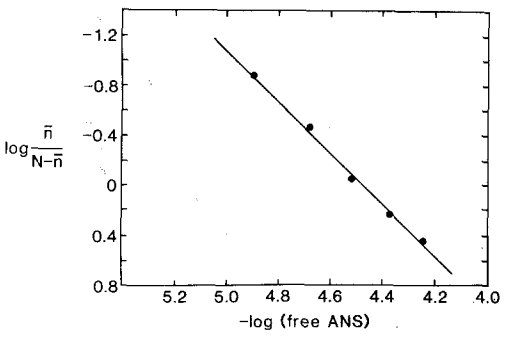


Figure 4. A Hill Plot of ANS Binding to Bovine Thyroglobulin - Data were taken from fluorescence titration (see Figure 3). N, the maximum number of binding sites, was taken as 6 (based on Scatchard analyses).

per thyroglobulin molecule (Figure 3) and some degree of cooperativity (since the binding occurs over a range of free ligand concentration less than that expected for independent sites). In order to confirm this binding, a series of equilibrium dialysis experiments were carried out. The results, also shown in Figure 3 (open circles), were in close agreement with the fluorescence titration data. A Hill plot of the binding data is shown in Figure 4. The Hill coefficient calculated from Figure 4 was 2.1, indicating moderate cooperativity between the ANS binding sites on the bovine thyroglobulin (15).

Discussion

Bovine thyroglobulin contains discrete binding sites for the fluorescent probe 1,8-anilinonaphthalene sulfonate. Six ANS molecules are bound per molecule of the protein at pH 7.0 in 0.1 M phosphate buffer with a moderate affinity (K_D = 3 x 10^{-5} M). The spectra shift and increased quantum yield of the fluorescence associated with the binding of ANS to thyroglobulin suggest nonpolar character at the ANS binding sites (16,17), although it is not possible to completely rule out the possibility of binding at a relatively polar site or sites where solvent orientation, such as that recently described by Johnson et al. (18), occurs.

The positive cooperativity observed in this binding phenomenon suggests that the ANS binding sites are either in close proximity to one another or that they are conformationally linked. While the significance of the ANS binding sites in thyroglobulin must be further defined, the sites represent reference points which may aid in the definition of structure and function of this complex iodinated glycoprotein. Since thyroid peroxidase, which catalyzes both the iodination and coupling reactions necessary to produce thyroid hormone, is a membrane associated enzyme, it is tempting to speculate that nonpolar sites or domains may play a role in thyroglobulin-thyroid peroxidase interaction.

The effects of ANS on the state of association of the polypeptides of thyroglobulin as well as its effect, if any, on the in vitro iodination and coupling reactions and hormonogenesis are under current investigation.

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