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Thyroglobulin Structure-Function: The Effect of Iodination on the Structure of Human Thyroglobulin

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SUMMARY: Thyroglobulin of very low iodine content has been prepared from a single non-toxic human goitre. The initial iodine content of the protein (0.038%) has been increased to levels of 0.16% and 0.85% by in vitro treatment with thyroid peroxidase and the resulting proteins studied with respect to their intrinsic fluorescence, circular dichroism spectra and binding of the hydrophobic probe 1,8-anilinonaphthalene sulfonic acid (ANS). While significant differences were observed between levels of iodination in both the ANS binding and intrinsic fluorescence of the thyroglobulin, no significant differences in the near and far UV circular dichroism spectra of the protein as a function of iodine content were observed. These data suggest that, the iodination of thyroglobulin effects specific areas of the protein without significant disruption of its overall secondary structure.

Thyroglobulin, the large glycoprotein found in the follicular lumen of the thyroid is the substrate for thyroid peroxidase (1), a membrane bound enzyme capable of iodination of tyrosine residues. As a limited number of tyrosine residues within this protein are iodinated (2) (3) and subsequently coupled to form thyroid hormone, several changes in the gross physical properties of the thyroglobulin have been reported. These changes include an increased sedimentation coefficient (4), an altered shape in electron micrographs (5) and altered dye binding (6). In addition, it has been suggested that the degree of iodination of thyroglobulin may alter its suceptibility to proteolytic digestion (7). Recently, Turner et al. have reported, using enzymatically iodinated thyroglobulin, that only very small amounts of thyroxine are produced at or below a level of 12 gram-atoms of iodine per mole thyroglobulin whereas about this level, a dramatic increase in thyroxine production occurs (8). These findings are consistent with a structural change in the thyroglobulin, at least at those sites responsible for hormone formation, when the degree of iodination increases to levels which exceed $\sim$12 g.a. per mole of protein. In order to determine whether the increased level of hormone formation seen above $\sim$12 g.a. of iodine per mole of thyroglobulin was due to a general or localized change in the protein structure, we have used thyroglobulin from a single goitrous gland and produced

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thyroglobulin samples with iodine contents above (44 g.a.) and below (8 g.a.) that level. Thus comparative measurements were possible on samples of the same protein preparation, varying only in extent of iodination from ~ 2 g.a. to ~ 44 g.a. per mole. Measurements of intrinsic fluorescence, and circular dichroism, as well as spectrofluorometric titrations using 1.8-anilinonaphthalene sulfonate (ANS) as a probe for nonpolar regions were carried out on the thyroglobulins of varying iodine content.

MATERIALS AND METHODS

Thyroglobulin Preparation. All thyroglobulin used in these experiments was extracted from a single non-toxic colloidal goiter and purified by ammonium sulfate fractionation and repeated gel filtration through Bio-Gel A-5m, 200-400 mesh (Bio-Rad Laboratories) (2,3). The iodine content of the isolated protein was determined to be 0.038% (N2g.a. I/mole Tg) using the spectrophotometric method of Sandell and Kolthoff (9). Samples of the purified thyroglobulin (1 mg/ml) were enzymatically iodinated at 37°C in a mixture containing hog thyroid peroxidase (5 mg/ml), glucose (1 mg/ml), glucose oxidase (39 mU/ml), and ^{127}I-iodide with a trace amount of ^{131}I-iodide (8). The incubation reaction was initiated by addition of glucose oxidase and was halted after the desired interval by rapid cooling of the tubes and the addition of methimazole (final concentration, 5 mM). Samples of thyroglobulin containing 8.3 and 44.3 g.a. I/mole Tg were prepared by varying the incubation times. The level of iodination was calculated from the specific activity of the ^{131}I solution, the fraction of added ^{131}I bound to the protein and the thyroglobulin concentration. Thyroglobulin was incubated as above, but omitting the thyroid peroxidase as one control. Unincubated thyroglobulin was used as an additional control.

All thyroglobulin samples were diluted to a concentration of 0.52 +/- 0.01 mg/ml (0.8 mM) with 0.1 M sodium phosphate buffer, adjusted to pH 7.0. Protein concentrations were determined spectrophotometrically on a Hitachi model 100-40 spectrophotometer using $E^{1\%}_{1cm} = 10$. Circular dichroism analysis was performed on each sample without further modification using a JASCO model J500-A spectropolarimeter. A pathlength of 2 cm was used while scanning the near UV spectrum and 1 cm when performing scans in the far UV range. All samples were scanned at least twice along with a buffer baseline.

Absorption spectra were obtained using a Cary 118 recording spectrophotometer. All samples were scanned in 1 cm quartz cuvettes at a full scale deflection of 1.0 absorbance units. Intrinsic emission and excitation fluorescence spectra were obtained from each thyroglobulin sample using a Perkin-Elmer MPF-44A spectrofluorometer. Emission spectra were recorded while exciting at 280 nm; during excitation scans emission was monitored at 331 nm. Monochromator slit widths were 5 nm for both emission and excitation light paths.

Fluorescence titrations with 1,8-Anilinonaphthalene sulfonate were carried out as previously described (10) using excitation at 366 nm and quinine sulfate as a quantuim yield reference. Data were treated according to Weber and Young (11), 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

Circular dichroism spectra of low iodine (~ 8g.a./mole), high iodine (~ 44g.a./mole) and control (~ 2g.a./mole) thyroglobulins showed very small differences in the range of 200-350 nm (Figure 1). Between 250 and 260 nm, the thyroglobulin containing the largest amount of iodine (44g.a./mole) had a slight decrease in ellipticity when compared to the

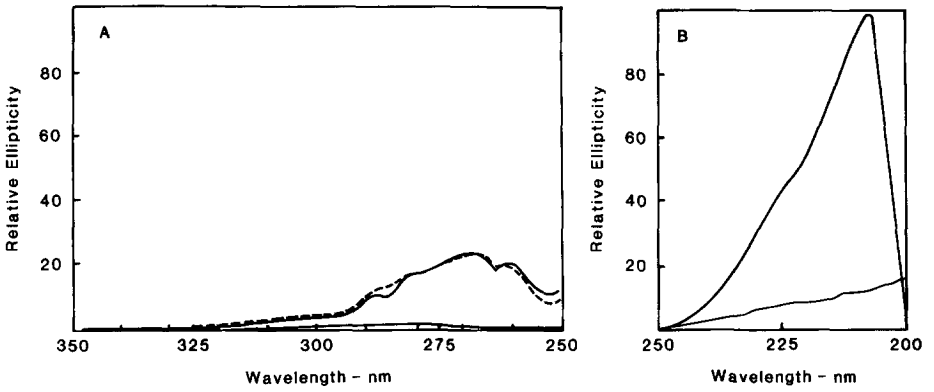

Figure 1. Circular Dichroism spectra-the control thyroglobulin sample is shown as a broken line and the buffer baseline is also indicated in the plots (A) - in the near U.V. region the control and low iodine samples were indistinguishable and are represented as a single broken line while the high iodine sample is shown as a solid line. (B) - the spectra of the samples in the far U.V. region were all identical and are shown as a single solid line.

samples containing less iodine, where as at ~ 285nm it showed a slight increase. These differences were, however, judged to be within the experimental error of the system. In the far UV between 200 and 250 nm the spectra were identical. In these experiments as well as in the other spectral experiments the data for the glucose/glucose oxidase incubated thyroglobulin control and unincubated thyroglobulin (starting material) were superimposible and are shown as a single line in Figures.

Inspection of absorbance spectra showed only small reduction in the region of 260-280 nm between the control and the lower level of iodination (Figure 2). The high iodine

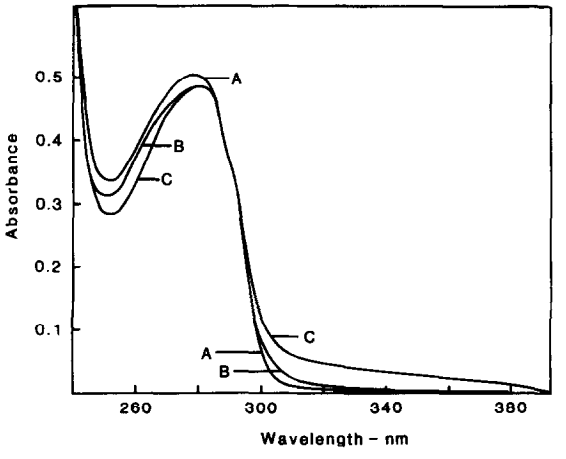

Figure 2. Absorption spectra - protein samples were carefully matched in concentration according to dry weight. The samples were dialyzed, lyophilized and weighed in parallel to control for salt and moisture content. A - controls (untreated and glucose oxidase treated), B - 8 g.a. I/mole, C - 44 g.a. I/mole.

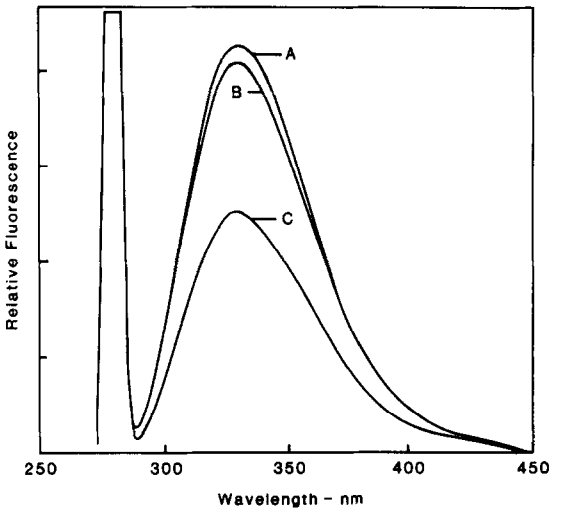

Figure 3. Intrinsic Fluorescence Spectra - excitation of the sample was at 280 nm with a 5nm bandpass employed in both the excitation and emission monochromators. A - controls (untreated and glucose oxidase treated), B- 8 g.a. I/mole, C - 44 g.a. I/mole.

sample, however, showed a further decrease absorbance near 250 nm and an increased absorbance in the region of 300-400 nm but no further decrease in absorption near 280 nm. These differences were attributed to the conversion of a fraction of the tyrosine residues in the protein to iodotyrosine derivatives which would be expected to show increased absorption between 300 and 400 nm (12) as well as a decrease in the area of 280 nm.

Intrinsic fluorescence emission spectra for each of the samples are shown in Figure 3. The emission maximum was at 331 nm in all cases. Analyses of the excitation spectra of these samples (not shown) showed no shifts in the excitation maximum between the various samples. While a small (less than 5%) reduction in the intrinsic fluorescence of the low iodine sample ($\sim$ 8g.a./mole) was observed with respect to the control or starting material, a 40% reduction in fluorescence emission was seen in the high iodine sample. In all cases, the intrinsic fluorescence emission was characteristic of tryptophan.

Each iodinated and control sample was titrated with ANS and the emission spectra were recorded along with those from parallel titrations of quinine sulfate as an optical and quantum yield reference. The emission maximum of the bound ANS was between 450 and 460 nm in all cases. A slight red shift (between 3 and 5 nm) was noted as the ANS concentration was increased. Titration data in the form of Scatchard plots are shown in

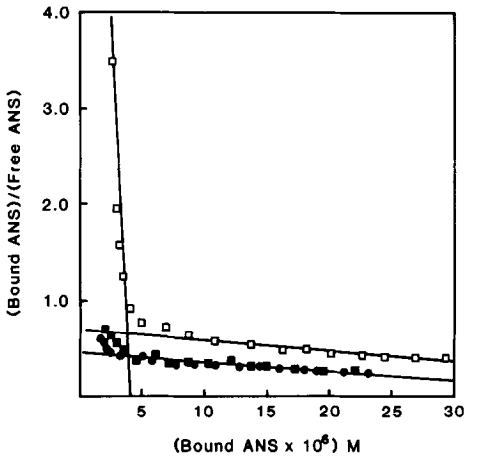

Figure 4. A Scatchard Plot of ANS Binding Data - the lines were calculated from linear regression analyses of the points in each region of the plot. Filled circles - control samples, filled squares - 8 g.a. I/mole, open squares - 44 g.a. I/mole.

Figure 4. The data obtained for the starting material and the low iodine sample indicated a large number (~90) binding sites of low affinity ($K_D \simeq 1 \times 10^{-4}$M). The high iodine sample, on the other hand, showed two clear classes of ANS binding sites: a high affinity class ($K_D \simeq 7 \times 10^{-7}$) with a stoichiometry of ~6 ANS bound per thyroglobulin molecule and a low affinity, high capacity class similar to that seen in the low iodine sample and control. Analysis of the binding data using Hill plots (not shown) indicated no significant cooperativity in the binding for either class of site.

DISCUSSION

While there is no doubt that some of the physical properties of thyroglobulin are related to the degree of iodination of the protein, the degree to which a change in the overall secondary and tertiary structure of the molecule may change with iodination has not been previously defined. The high iodine sample studied here had an iodine content within the normal range for human thyroglobilin. Circular dichroism data are consistent with conservation of the basic secondary and tertiary structure of thyroglobulin as iodination proceeds from a very low level to that found in the normal protein. Therefore the altered characteristics of thyroglobulins as a function of level of iodination are likely to stem from a combination of changes in density (partial specific volume), very restricted or limited conformational alteration and subunit rearrangement. The changes seen in the absorption spectrum of the protein as iodination increases can be attributed

to the conversion of tyrosine residues to iodotyrosine, iodothyronine and, at least to a small extent, dehydroalanine residues (12)(13).

While some quenching of the intrinsic fluorescence of the protein would be expected as a result of a direct effect of iodination of tyrosine residues (due to the conversion of tyrosine to an essentially non fluorescent iododerivative) the extent of quenching observed in the high iodine sample substantially exceeded the expected effect. Since only ~ 25 of nearly 130 tyrosines are iodinated under these conditions (14) and the principal emission is derived from the ~ 90 tryptophan residues in the protein, the 40% reduction observed in the intrinsic fluorescence of the high iodine sample clearly reflects indirect effects. The most likely causes of this significant additional reduction in quantum yield are reduced energy transfer from tyrosine to tryptophan due to a decrease in potentially fluorescent tyrosines and a further depopulation of tyrosine excited states by energy transfer to monoidotyrosine (absorption maximum near 305 nm) as well as transfer from excited tryptophan residues to non-fluorescent diiodotyrosine and iodothyronine derivatives with significant absorption in the spectral region of tryptophan emission (300-350 nm).

Human thyroglobulin iodinated to 44 g.a.I/mole was found to contain six discrete, high affinity binding sites ($K_D \simeq 1 \times 10^{-}$ M). These findings are somewhat difficult to relate to the report from Palumbo and Ambrosio (6) who observed a decreased binding stoichimetry with increased levels of iodination and a single class of binding site in porcine thyroglobulin subfractions which had been prepared by density gradient procedures. It should be pointed out that, in addition to the difference in species, Palumbo and Ambrosio carried out binding over an ANS concentration range which was significantly below the K_D for the lower affinity site class reported here. In addition, the iodine levels of the samples used by these authors were above those reported here. In an earlier report (10), we have shown that normally iodinated bovine thyroglobulin contains 6 binding sites for ANS of intermediate affinity ($K_D \simeq 3 \times 10^{-5}$M).

In summary, we have examined samples of human thyroglobulin derived from an identical starting protein. The initial material with an iodine content of ~ 2 g.a./mole was iodinated, using an <u>in vitro</u> thyroid peroxidase procedure, to levels of ~ 8g.a./mole (low iodine sample) and ~ 44g.a./mole (high iodine sample). While circular dichroism

measurements suggest no major change in the overall conformation of human thyroglobulin as it is iodinated to at least 0.8%, both intrinsic fluorescence and high affinity ANS binding are effected by increased iodination, with the former being significantly diminished and the later increased.

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