MODIFICATION OF THYROXINE-BINDING GLOBULIN WITH *p*-IODOPHENYLSULFONYL (PIPSYL) CHLORIDE AND EFFECT ON THYROXINE BINDING ACTIVITY

Jessie Shih SIEGEL, Ladislav KORCEK and Milton TABACHNICK
Department of Biochemistry, New York Medical College, Valhalla, NY 10595, USA

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1. Introduction

Thyroxine-binding globulin (TBG) is the major transport protein of the thyroid hormones in human plasma. As part of a study of the chemistry of the thyroxine binding site on TBG, we have been investigating a series of protein modifying reagents for their effect on thyroxine binding. Among the reagents assayed, *p*-iodophenylsulfonyl (pipsyl) chloride proved to be of interest, since relatively low levels of protein modification with this reagent resulted in concomitant decreases in thyroxine binding activity. The present communication describes the results of an investigation involving the effect of derivatization of TBG with pipsyl chloride on the thyroxine binding activity of the protein. The results indicate that there is a direct relationship between the degree of pipsylation and the percent decrease in thyroxine binding activity at relatively low levels of pipsyl group incorporated per mole protein. Analyses of hydrolyzates of pipsylated TBG preparations modified with [^{35}S]-pipsyl chloride, indicate that the principal group derivatized in TBG is an ϵ-amino group of a specific lysyl residue probably located near the thyroxine binding site.

2. Materials and methods

Thyroxine-binding globulin (TBG) was isolated from human plasma, as in [1]. TBG solutions were concentrated by ultrafiltration to ~10 mg/ml final conc. TBG preparations obtained from 3 separate purifications were pooled and stored at −20°C prior to use. Based on the iodine content, the TBG contained an average of 0.2 mol residually bound thyroxine/mol protein. TBG was taken as mol. wt 60 000 [2].

^{35}S-Labeled *p*-iodophenylsulfonyl chloride (123 mCi/mmol) in solid form and ^{125}I-labeled thyroxine (38 Ci/mmol) in 50% aqueous propylene glycol were obtained from Amersham Corporation. Unlabeled *p*-iodophenylsulfonyl chloride was recrystallized 3 times from acetone–water and the final product was dried over P_2O_5 in vacuo. Unlabeled L-thyroxine was obtained from Aldrich Chemical Company and tetraiodothyroacetic acid was obtained from Vega-Fox Biochemicals.

ϵ-*N*-Pipsyllysine was synthesized by the method in [3] and pipsylalanine and pipsylglycine were synthesized as in [4].

Before use, the [^{35}S]pipsyl chloride was purified by thin-layer chromatography (TLC) on prewashed activated Silica gel plates, 250 μ (Analtech), in chloroform–ethyl acetate–formic acid (5:4:1, v/v/v). Pipsyl chloride was detected with ultraviolet light at 254 nm. The spot containing radioactive pipsyl chloride was scraped off the plate and the [^{35}S]pipsyl chloride was extracted into acetone. Purified [^{35}S]-pipsyl chloride was mixed with unlabeled pipsyl chloride in acetone to give working radioactive solutions containing 4–8 μmol/ml of [^{35}S]pipsyl chloride (spec. act. 10–50 mCi/mmol). Only freshly purified [^{35}S]pipsyl chloride was used in the coupling experiments with TBG.

In a typical experiment, [^{35}S]pipsyl chloride (40 nmol, 1.4×10^6 cpm) in 10 μl acetone was added to ~500 μg (8 nmol) of TBG in 0.25 ml 0.08 M borate buffer (pH 9.0). Differentially pipsylated TBG samples were prepared by varying the molar ratios of

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[^{35}S]pipsyl chloride to TBG in the reaction mixture. The reaction was allowed to proceed in the dark at 0°C for 2 h. To terminate the reaction, 0.2 ml 0.3 M potassium phosphate buffer (pH 7.4) was added to the mixture bringing it to pH 7.8 and the solution was chromatographed on Sephadex G-25 (fine) to separate [^{35}S]pipsylated-TBG from unreacted [^{35}S]-pipsyl chloride. The elution buffer was 0.06 M potassium phosphate (pH 7.4) containing 0.02% sodium azide. The derivatized TBG emerges in the excluded volume, while unreacted [^{35}S]pipsyl chloride is eluted after 2.5 column volumes. The thyroxine binding activity of a pipsylated-TBG preparation was compared with that of underivatized TBG which had been subjected to the same procedure, but in the absence of [^{35}S]-pipsyl chloride. To compare binding activities, the bound/free value for binding of thyroxine to TBG or pipsylated TBG was determined by equilibrium dialysis at a fixed molar ratio of 0.26 of added [^{125}I]-thyroxine to protein. Equilibrium dialysis was performed at 25°C and pH 7.4 in 0.06 M potassium phosphate, 0.7 mM EDTA, as in [1].

Hydrolysis of [^{35}S]pipsyl-TBG (~600 μg) was done in 1 ml constantly boiling 6 N HCl in vacuo at 110°C for 20 h. To aid in detection, 25 μg unlabeled ε-*N*-pipsyllysine was added prior to hydrolysis. After taking the hydrolyzate to dryness under vacuum at 37°C, the residue was extracted with 0.5 ml acetone–methanol mixture (1:1, v/v). Aliquots of the material soluble in acetone–methanol were subjected to TLC in two solvents: chloroform–ethyl acetate–formic acid (5:4:1, v/v/v) on Silica gel 250 μ plates; butanol–acetic acid–water (4:1:2, v/v/v) on Silica gel F254 plates (Merck). The pipsyl amino acids were detected on the TLC plate under ultraviolet light at 254 nm. The limit of detection of a pipsyl amino acid, under ultraviolet light, was ~10 nmol (4 μg pipsyllysine). To determine the distribution of ^{35}S-radioactivity on the TLC plate, 0.5 cm portions of the silica gel were scraped off, added to scintillation cocktail and counted.

^{35}S-radioactivity was determined in a Nuclear-Chicago Isocap/300 Liquid Scintillation System with an efficiency of 80%. ^{125}I-radioactivity was measured in a Nuclear-Chicago 1185 System auto-gamma well-type scintillation counter. Protein was determined by the Lowry [5] procedure. Analysis of the iodine contents of TBG and pipsylated TBG was done by Boston Medical Laboratory.

3. Results

Determination of the degree of derivatization as a function of time indicated that the reaction of pipsyl chloride with TBG at 0°C and pH 9 was essentially complete in 2 h (fig.1). Apparently, there is little further derivatization of the protein after 2 h.

The binding activity of pipsylated TBG preparations was found to decrease progressively with the degree of pipsylation, as shown by fig.2, in which the bound/free value for a TBG sample has been plotted against moles pipsyl group incorporated per mole protein.

The experimental results given in fig.2 have been calculated on the basis of the binding activity of a pipsylated TBG preparation relative to that of underivatized TBG (bound/free = 20 in fig.2) and have been replotted in fig.3. Two additional points are also given in fig.3.

As shown in fig.3, the decrease in percent binding activity is directly related to the degree of pipsylation up to 1.22 mol pipsyl group incorporated/mol TBG. The correlation coefficient for the relationship between the percent loss in binding activity, as a function of the degree of pipsylation of TBG, is 0.994, based on a least squares fit of the data. Extrapolation of the straight line in fig.3 to the abscissa gives a limiting value of 1.26 mol pipsyl group bound/mol TBG

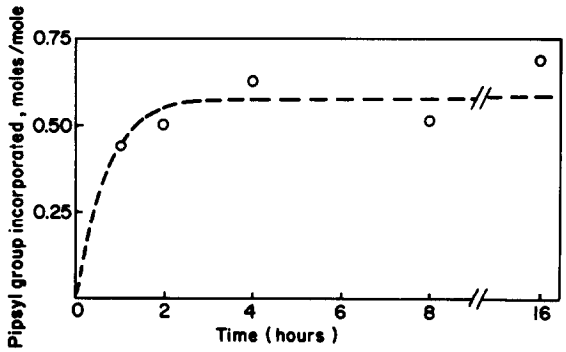

Fig.1. Modification of TBG with pipsyl chloride. Each point represents a separate reaction carried out at 0°C and pH 9. Each mixture contained TBG (500 μg, 8.3 nmol) and pipsyl chloride (33 nmol) in 0.25 ml 0.08 M borate buffer (pH 9). The degree of pipsylation is based on the iodine content of the derivatized TBG samples correcting for the iodide contribution of 0.2 mol residually bound thyroxine in the original TBG preparation.

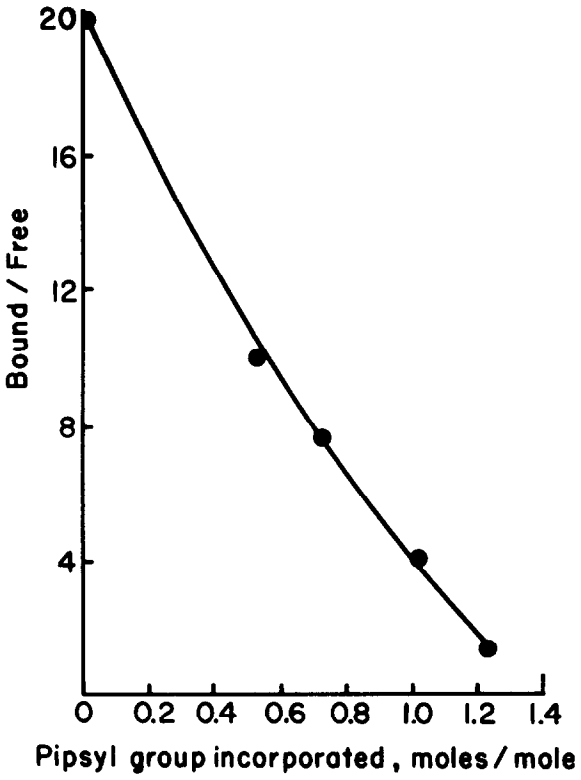

Fig 2. Effect of pipsylation of TBG on thyroxine binding activity.

at minimum percent binding activity. More highly derivatized TBG samples containing several moles of pipsyl group incorporated per mole protein were also prepared. At 3.4 mol pipsyl group/mol TBG there is still a small degree (~3%) of residual binding activity (fig.3) which falls to ~2% at 8.3 mol pipsyl group/mol TBG (data not shown).

To determine if modification of an amino acid side chain at the thyroxine binding site had taken place, [^{35}S]pipsyl chloride was reacted with TBG in the presence and absence of thyroxine. Thyroxine is bound to TBG at a single binding site [1] and at the molar ratio of added thyroxine to TBG, 1.2:1 (table 1), essentially all the thyroxine is bound at 0°C. As shown in table 1, there is a partial inhibition (33%) in the degree of derivatization in the presence of thyroxine. Since pipsyl chloride is capable of reacting with the α-amino group of thyroxine, a similar experiment was done, using an analogue lacking an α-amino group, tetraiodothyroacetic acid (5:1 molar ratio). As compared to the results obtained with thyroxine, pipsylation was inhibited 29% in the presence of tetraiodothyroacetic acid (table 1).

To identify the amino acid residue, or residues that might be derivatized, a sample of pipsylated TBG containing 0.96 mol [^{35}S]pipsyl group bound/mol

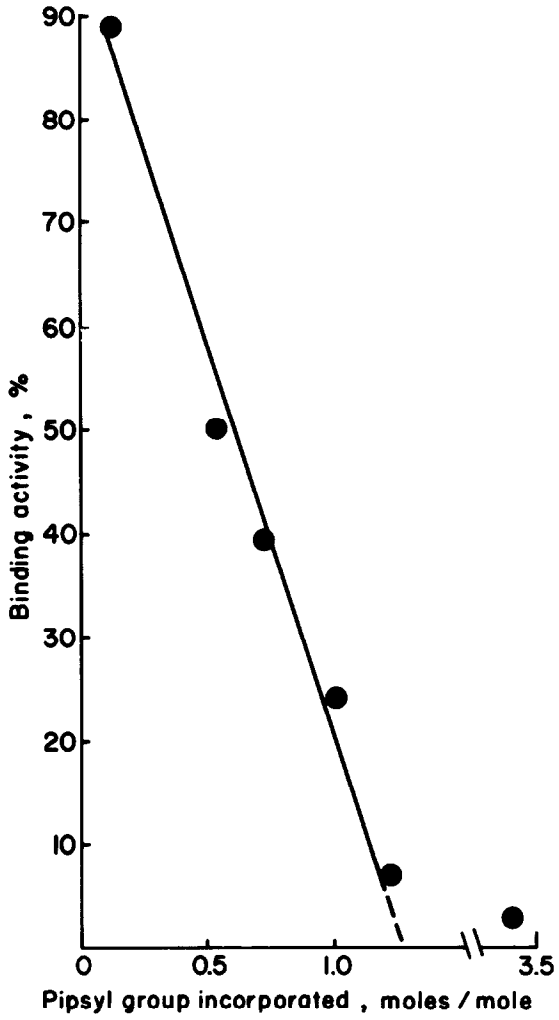

Fig.3. Decrease in percent binding activity of pipsylated TBG as a function of the degree of pipsylation. The 4 points, from 0.54–1.22 mol pipsyl group incorporated/mol TBG, are based on the data in fig.2. Two additional points, 0.13 mol and 3.4 mol pipsyl group/mol TBG are also included. The percent binding activity is defined as:

$$\frac{\text{bound/free, pipsylated TBG}}{\text{bound/free, underivatized TBG}} \times 100$$

Table 1
Partial reduction in the degree of modification of TBG by [^{35}S]pipsyl chloride in the presence of thyroxine and tetraiodothyroacetic acid

	[^{35}S]Pipsyl group incorporated into TBG		Decrease in extent of pipsylation
	(cpm/μg)	(mol/mol)	(%)
TBG + [^{35}S]pipsyl chloride[a]	963	1.03	
TBG + thyroxine + [^{35}S]pipsyl chloride	641	0.69	33
TBG + [^{35}S]pipsyl chloride[b]	264	0.99	
TBG + tetraiodothyroacetic acid + [^{35}S]pipsyl chloride	187	0.70	29

[a] TBG (0.5 mg, 8.3 nmol) was reacted with [^{35}S]pipsyl chloride (42 nmol, 2.35 × 10^6 cpm) for 2 h at 0°C at pH 9, in 0.25 ml 0.08 M borate buffer in the absence and presence of thyroxine (9.7 nmol)

[b] TBG (1 mg, 16.7 nmol) was reacted with [^{35}S]pipsyl chloride (83 nmol, 1.32 × 10^6 cpm) for 2 h at 0°C, at pH 9, in 0.30 ml 0.08 M borate buffer in the absence and presence of tetraiodothyroacetic acid (83.5 nmol)

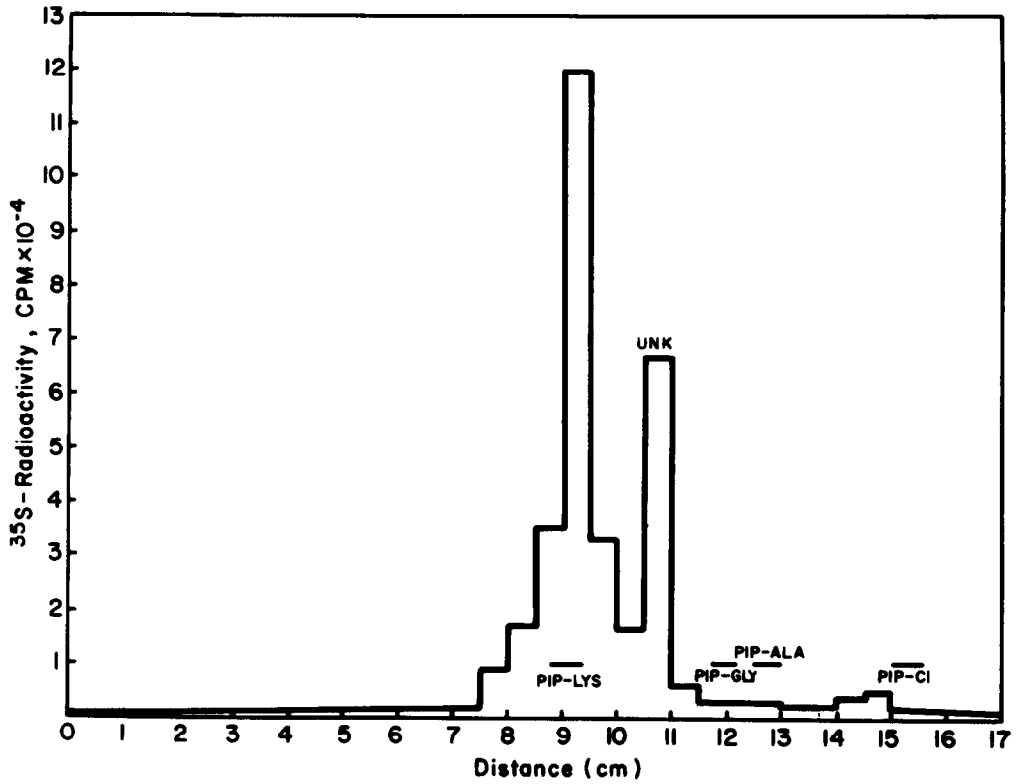

Fig.4. Thin-layer chromatogram of an extract of an hydrolyzate of pipsyl-TBG containing 0.96 mol [^{35}S]pipsyl group/mol protein. The solvent was *n*-butanol–acetic acid–water, 4:1:5 (v/v/v). PIP-LYS, ε-*N*-pipsyllysine; UNK, unknown; PIP-GLY, pipsylglycine; PIP-ALA, pipsylalanine; PIP-CL, pipsylchloride.

TBG was hydrolyzed and the distribution of ^{35}S was determined by TLC. About 70% of the radioactivity chromatographed identically with authentic ϵ-*N*-pipsyllysine, and ~25% of the radioactivity was concentrated in an unidentified spot that moved ahead of the ϵ-*N*-pipsyllysine in *n*-butanol–acetic acid–water (fig.4). A diagrammatic representation of a typical TLC result is shown in fig.4. In another solvent, chloroform–ethyl acetate–formic acid, a second sample of TBG, containing 1 mol [^{35}S]pipsyl group/mol TBG gave similar results: 65% of the radioactivity in the ϵ-*N*-pipsyllysine position (R_F 0.1) and 25% unidentified and remaining at the origin. Assuming a 89% recovery of ϵ-*N*-pipsyllysine after hydrolysis [6] the corrected average yield of ^{35}S-radioactivity as pipsyllysine would be 76%. The latter value is in very good agreement with the average loss of 75% in binding activity of the [^{35}S]pipsyl-TBG samples taken for hydrolysis and indicates that modification of lysine can account completely for the loss in binding activity.

4. Discussion

The close relationship between the degree of pipsylation and the percent loss in thyroxine binding activity, up to 1.2 mol pipsyl group incorporated/mol protein, suggests that a specific group in TBG has been modified. Based on the analytical results, the specific group derivatized appears to be an ϵ-amino group of lysine. Apparently little derivatization of the N-terminal alanyl residue [7] takes place at 1 mol pipsyl group incorporated/mol TBG.

The observation that 1.26 mol, instead of 1 mol pipsyl group incorporated/mol TBG is required to reach minimal binding activity, indicates that there is non-specific binding. Non-specific binding is known to occur, since TBG preparations containing 3.4 and 8.3 mol pipsyl group/mol protein can be prepared.

There is only a small reduction in the degree of pipsylation when the binding site on TBG is blocked with added thyroxine. This would suggest that the specifically pipsylated lysyl residue is not actually part of the thyroxine binding site but is more probably located sufficiently close to the binding site to permit the *p*-iodophenylsulfonyl group to extend over and block the site. It may be noted that the *p*-iodophenylsulfonyl group is a relatively rigid structure with a length of ~10 Å, based on Van der Waals' and covalent radii. An alternative explanation for the effect of pipsylation and the relatively small inhibitory effect of bound thryoxine is that at least 2 lysyl residues are involved, one is at the binding site and is ~30% derivatized, while the other is located near but not at the binding site, and is ~90–100% derivatized. This would account for the stoichiometry of 1.26:1. A third possibility is that the reactive lysyl residue is quite distant from the binding site and on derivatization with pipsyl chloride, a conformational change is induced in TBG leading to a decrease in thyroxine-binding activity. The available information does not permit a definite conclusion in favor of any single explanation to the exclusion of the others. Further work will be required before a more complete explanation can be made for the effect of pipsylation on the thyroxine-binding activity of TBG.

Acknowledgement

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References

[1] Korcek, L. and Tabachnick, M. (1976) J. Biol. Chem. 251, 3558–3562.
[2] Korcek, L. and Tabachnick, M. (1974) Biochim. Biophys. Acta 371, 323–336.
[3] Oratz, M., Burks, A. L. and Rothschild, M. A. (1966) Biochim. Biophys. Acta 115, 88–94.
[4] Keston, A. S., Udenfriend, S. and Cannan, R. K. (1949) J. Am. Chem. Soc. 71, 249–257.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–269.
[6] Udenfriend, S. and Velick, S. F. (1951) J. Biol. Chem. 190, 733–740.
[7] Cheng, S. Y. (1977) Biochem. Biophys. Res. Commun. 79, 1212–1218.