Affinity Labeling of the Thyroxine Binding Domain of Human Serum Prealbumin with Dansyl Chloride†

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ABSTRACT: The binding of thyroxine to prealbumin was almost completely abolished when the latter had been treated with 2 mol of dansyl chloride/mol. When dansyl chloride was replaced with dansylglycine, the latter bound noncovalently to prealbumin (n = 2.0; k = 2.9 × 10^5 M^{-1}; α = 0.80). Competitive equilibrium dialysis as well as spectrophotometric titration showed that dansylglycine and thyroxine compete for the same binding domain on prealbumin. Dansyl chloride is therefore an affinity label for the localization of the thyroxine binding sites on prealbumin. Acid hydrolysis of dansyl prealbumin containing 1.7 tritiated dansyl residues/mol gave N^{ϵ}-dansyllysine as the only labeled amino acid. Various fractionation procedures showed that the labeled residue is lysine-15. This lysine which is located in the central channel formed by the four subunits of prealbumin is therefore within the dansyl and thyroxine binding domain of prealbumin.

PA[1] is involved in the transport of the thyroid hormones and of vitamin A (retinol) in plasma. While PA binds T_4 and triiodothyronine directly, its binding to vitamin A is mediated by another transport protein, RBP. Vitamin A is bound to RBP which in turn is bound to PA (Kanai et al., 1968). The latter has two binding sites for T_4 (Ferguson et al., 1975) and four for RBP (Van Jaarsveld et al., 1973).

PA is a tetramer composed of four identical subunits of molecular weight ~13,700 whose complete amino acid sequence has recently been elucidated (Kanda et al., 1974). The subunits have the overall shape of elongated cylinders and are tetrahedrally arranged in such a manner that a central channel is formed which penetrates the entire molecule (Blake et al., 1974). This structure is unusual since other carrier proteins or enzymes whose structures have been elucidated have crevasses rather than channels. The four binding sites for RBP on PA are best explained by assuming that each subunit carries one ligand. The fact that PA has only two binding sites for T_4 suggests that these are located inside the central channel. This has indeed been confirmed by X-ray analysis (Blake et al., 1974).

The aim of our studies is to localize the site of attachment of T_4 within the channel by the method of affinity labeling. Since it was found in our laboratory that ANS binds to the same two sites as T_4 (Ferguson et al., 1975) we selected a structurally closely related compound, Dns-Cl, as a possible affinity label. Dns-Cl, in contrast to ANS, has a reactive sulfonyl chloride group and is therefore capable of establishing a covalent bond with a nearby nucleophile. We established first by spectrophotometric titration and by equilibrium dialysis experiments that the DNS group, like ANS, binds to the same sites as T_4. We could then show by various fragmentation and fractionation procedures that Dns-Cl attaches itself to two of the four Lys-15 residues of PA.

Methods and Materials

Human serum PA was obtained from Behring Diagnostics and purified by preparative gel electrophoresis at pH 8.3 (50 mM Tris-glycine) using a gel containing 8.5% acrylamide and 0.18% N,N'-methylenebisacrylamide. PA concentrations were determined spectrophotometrically at 280 nm ($E_{1\ cm}$ (1%) 14.1) (Raz and Goodman, 1969).

L-T_4 and [^{125}I]-L-T_4 were the same as described previously (Ferguson et al., 1975).

Dns-Cl (Aldrich) was purified by recrystallization from isooctane–acetone (20:3). [*methyl*-^{3}H]Dns-Cl (in acetone, 1.81 Ci/mmol) was from New England Nuclear. The concentration of [^{3}H]Dns-Cl was determined from its molar extinction in acetone at 369 nm (ϵ 3690) (Gray, 1972a). Dns-Gly (Sigma) was recrystallized as the piperidinium salt from ethyl acetate and its purity assayed by thin-layer chromatography (TLC) in the following solvent systems: chloroform–methanol–acetic acid (45:4:1 and 15:5:1); upper phase of ethyl acetate–methanol–2M NH_4OH (5:2:3). The R_f values were 0.27, 0.74, and 0.54, respectively, with silica gel plates Q1F of Quantum Industries, Fairfield, N.J. [^{3}H]Dns-Gly was prepared by mixing equal volumes of 10^{-3} M [^{3}H]Dns-Cl (250 Ci/mol) in acetone and 2 × 10^{-3} M glycine in 0.1 M $NaHCO_3$ (pH 8.8). After 2 hr the reaction product was extracted into ethyl acetate and purified by preparative TLC on silica gel plates. Analytical TLC revealed a single spot.

Trypsin treated with L-tosylamido-2-phenylethyl methyl ketone was obtained from Worthington.

Binding Studies with Dns-Gly. Spectrophotometric titrations were carried out by adding Dns-Gly in small increments to a solution of PA. This was followed by the stepwise addition of T_4. Difference spectra of free and PA-bound Dns-Gly were determined using the tandem technique (Herskovits and Laskowski, 1962). Equilibrium dialysis was done as described previously for ANS (Ferguson et al., 1975). Dns-PA was prepared as described below under Affinity Labeling of PA with [^{3}H]Dns-Cl, except that the charcoal and guanidine treatments were omitted. The dan-

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[1] Abbreviations used are: PA, prealbumin; T_4, thyroxine; Dns, 5-dimethylamino-1-naphthalenesulfonyl (dansyl); ANS, 8-anilino-1-naphthalenesulfonic acid; RBP, retinol-binding protein.

sylated PA was merely dialyzed extensively against the buffer used in the equilibrium dialysis experiments. The curves shown in Figures 2 and 3 have been generated using the interacting sites model of Ferguson et al. (1975) and the following binding parameters for T_4: $k = 5.5 \times 10^7\ M^{-1}$, $\alpha = 0.041$, and $n = 2.0$.

Amino acid analyses were performed according to Spackman et al. (1958). Hydrolysis of the samples was carried out with double-distilled constant-boiling HCl in evacuated, sealed tubes at 105° for 22 hr.

End group determinations using Dns-Cl were performed as described by Gray (1972b) and manual Edman degradations according to Sauer et al. (1974) except that dimethylallylamine was replaced with dimethylbenzylamine. Two-dimensional TLC was used for the separation and identification of Dns-amino acids (Hartley, 1970).

Tritium was counted in Aquasol (New England Nuclear).

Affinity Labeling of PA with [^{3}H]Dns-Cl. To an ice-cooled solution of PA (8.4 mg) in 9.1 ml of 0.1 *M* $NaHCO_3$ buffer (pH 8.8) was added 0.9 ml of a 0.01% solution of [^{3}H]Dns-Cl (1.81 Ci/mmol) in acetone. The reaction mixture was stirred at 0° for 50 min. Then 1 ml of 2.5% dextran-coated charcoal was added and stirring at 0° was continued for 30 min. After centrifugation at 35,000*g* (4°, 30 min), the supernatant was dialyzed at room temperature against 6 *M* guanidine hydrochloride (pH 3.6) for 40 hr and then against H_2O for 3 hr. The slightly turbid dialysate was clarified by the addition of 1 μl of 2.5 *M* NaOH.

Tryptic Digestion of Maleylated Dns-PA. Maleylation of Dns-PA was based on the method of Butler et al. (1969, 1972). A solution of 8.5 mg of Dns-PA in 6 *M* guanidine hydrochloride (pH 3.6) was kept at room temperature for 72 hr. Solid $Na_4P_2O_7 \cdot 10\ H_2O$ (57 mg) was then added and the pH adjusted with NaOH to ~8.9. Solid resublimed maleic anhydride (7 mg) was added in several portions and the pH maintained between 8 and 9 by the addition of 20% NaOH. After 50 min the solution was dialyzed at room temperature against 0.1 *M* NH_4HCO_3 buffer (pH 8.5) for 4 hr with four changes.

After lyophilization, the maleylated Dns-PA was dissolved in 0.5 ml of the same buffer and the solution was digested with trypsin for 6 hr at 37° (Dns-PA/trypsin weight ratio, 50:1).

Column Fractionation. The tryptic digest was applied to a Sephadex column (G-50, fine, 1.2 × 200 cm) and the peptides were eluted with 0.1 *M* NH_4HCO_3 buffer (pH 8.5). The fractions comprising the major radioactive peak were pooled, lyophilized, and, after demaleylation in 5% HCOOH for 24 hr at 45°, rechromatographed on a 0.6 × 20 cm column of the cation exchange resin AG50W-X4, 30–35 μ (Bio-Rad) at 50°. In this fractionation gradient elution from 0.2 *M* acetic acid–pyridine (pH 3.1) to 2 *M* acetic acid–pyridine (pH 5.1) (molarity based on pyridine) was used (Kanda et al., 1974).

Cleavage with CNBr. The pooled fractions under the major radioactive peak from the ion exchange chromatographic fractionation were lyophilized. A solution of the residue in 50 μl of 0.1 *M* HCl was treated at room temperature with 2–3 mg of CNBr. After 18 hr the reaction mixture was evaporated to dryness.

Dns-PA was also treated directly with CNBr without prior maleylation and tryptic digestion. [^{3}H]Dns-PA (1 mg) was dissolved in 130 μl of 6 *M* guanidine in 0.3 *M* HCl. This solution was treated with 2–3 mg of CNBr as above except that the reaction mixture was dialyzed before evaporation.

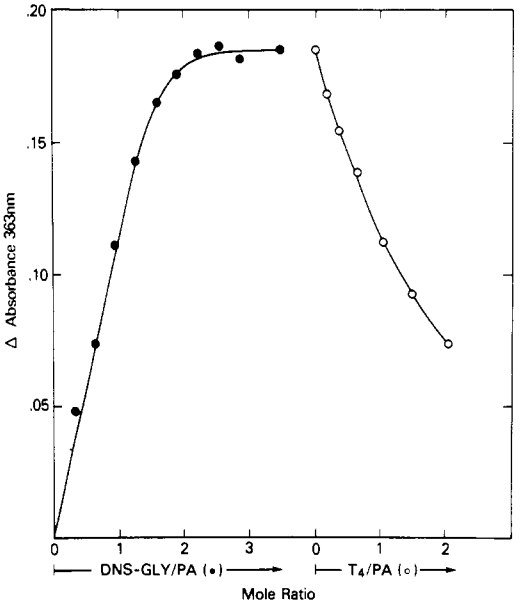

FIGURE 1: Spectrophotometric titration of PA with Dns-Gly, followed by L-T_4. The difference absorption of PA and Dns-Gly-PA at 363 nm is plotted against the mole ratio Dns-Gly/PA (●) or T_4/PA (○). PA concentration at the start of the titration, $9.78 \times 10^{-5}\ M$; after the addition of Dns-Gly and before that of T_4, $9.27 \times 10^{-5}\ M$; after the addition of T_4, $8.39 \times 10^{-5}\ M$. Solvent, 0.1 *M* sodium phosphate buffer (pH 7.4) at 25 ± 1°. The T_4/PA molar ratio could not be increased beyond 2 on account of the low solubility of T_4. The nonlinearity of the T_4 titration curve is due to the strong negative cooperativity between the two binding sites for T_4 (Ferguson et al., 1975) and to the competition of T_4 with Dns-Gly for the second site.

Results

Spectrophotometric Titrations. The uv spectra of Dns-Gly in the presence and absence of PA have a maximal difference at 363 nm. The existence of a difference peak shows that Dns-Gly binds to PA. A gradual increase in the Dns-Gly/PA ratio results in a corresponding increase in the size of the difference peak until saturation is reached at a ratio of about 2. When T_4 is added to a solution of PA, which is saturated with Dns-Gly, the size of the difference peak decreases with increasing T_4/PA ratio (Figure 1). The spectrophotometric titration data indicate two binding sites for Dns-Gly and the displacement of bound Dns-Gly from these sites by T_4. Thus, the behavior of Dns-Gly is similar to that of ANS (Ferguson et al., 1975).

Equilibrium Dialysis. The binding of T_4 to PA is nearly abolished after treatment of PA with 2 mol of Dns-Cl/mol (Figure 2). This suggests that the covalent binding of Dns has taken place at or near the binding site for T_4. In Figure 3 the equilibrium dialysis data for T_4 and PA in the presence of Dns-Gly are given. The good fit of the data to the model shows that Dns-Gly and T_4 compete for the same site on PA. The same figure also shows the binding data for T_4 in the absence of Dns-Gly and those for Dns-Gly in the absence of T_4. The interactive model of Ferguson et al. (1975) gives the following binding parameters for Dns-Gly: $n = 2.0$; $k = 2.9 \times 10^5\ M^{-1}$; $\alpha = 0.80$. The association constant is considerably lower than that of T_4 yet high enough for Dns-Cl to be used as an efficient affinity label (assuming $k_{\text{Dns-Cl}} \simeq k_{\text{Dns-Gly}}$).

Affinity Labeling of PA with [^{3}H]Dns-Cl. The molar

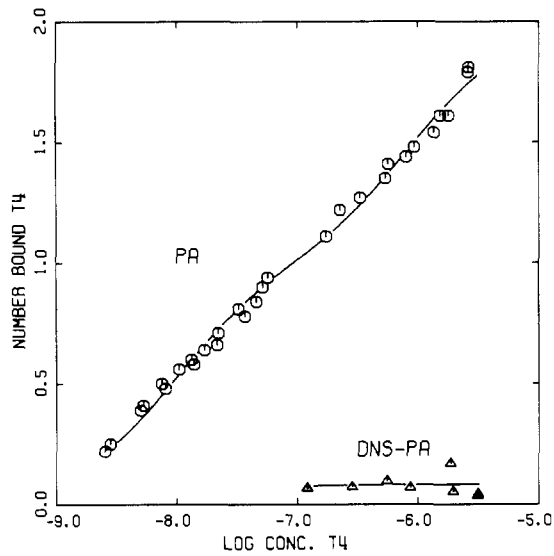

FIGURE 2: Binding of [^{125}I]-L-T_4 to PA and to Dns-PA, determined by equilibrium dialysis. The mole ratio T_4 bound/PA (○) or T_4 bound/Dns-PA (△) is plotted against the log concentration of free T_4. PA concentration, 0.50–0.94 μM; Dns-PA concentration (1.8 Dns residues/PA), 0.86 μM. The points are experimental data; the lines are calculated curves (cf. eq 2 of Ferguson et al., 1975).

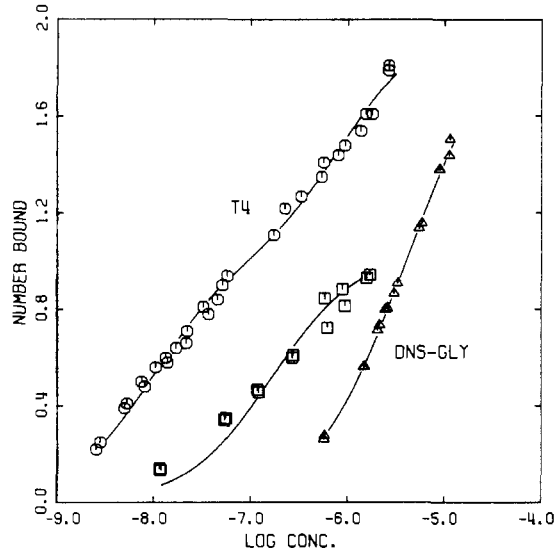

FIGURE 3: Binding of [^{125}I]-L-T_4 to PA in the presence of Dns-Gly (□), determined by equilibrium dialysis; Dns-Gly bound/PA is plotted against the log concentration of free T_4. Data for [^{125}I]-L-T_4 in the absence of Dns-Gly (○) and for [^{3}H]Dns-Gly in the absence of T_4 (△) are shown for comparison. In these cases the abscissa refers to the log concentration of free T_4 and of free Dns-Gly, respectively; PA concentration, 0.50–0.94 μM. The points are experimental data; the lines are calculated curves (cf. eq 2 and 4 of Ferguson et al., 1975).

Dns-Cl/PA ratio used for affinity labeling was 2.1:1. After removal of free Dns-OH by treatment with dextran-coated charcoal, followed by thorough dialysis against 6 M guanidine, 80% of the original radioactivity remained associated with the protein. Acid hydrolysis of the labeled protein containing 1.7 mol of Dns/mol of PA gave a single Dns-amino acid which was identified by two-dimensional TLC as N^{ϵ}-Dns-Lys. The chromatogram showed in addition a spot of Dns-OH representing 15–20% of the radioactivity.

Determination of the Site of Attachment of Dns to PA. Maleylation of the Dns-labeled PA resulted in the blocking of all free ϵ-amino groups of the Lys residues of PA as

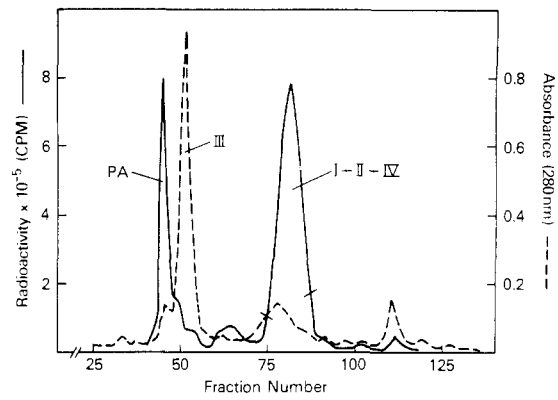

FIGURE 4: Elution of the tryptic digest of maleylated [^{3}H]Dns-PA from a Sephadex G-50 column (1.2 × 200 cm). Eluent, 0.1 M NH_4HCO_3 buffer (pH 8.5); temperature, 25°, flow rate, 4.5 ml/hr; fraction size, 1.5 ml. The roman numbers refer to the four peptides present in the tryptic digest (see Results). The fractions between slash marks were pooled for further treatment.

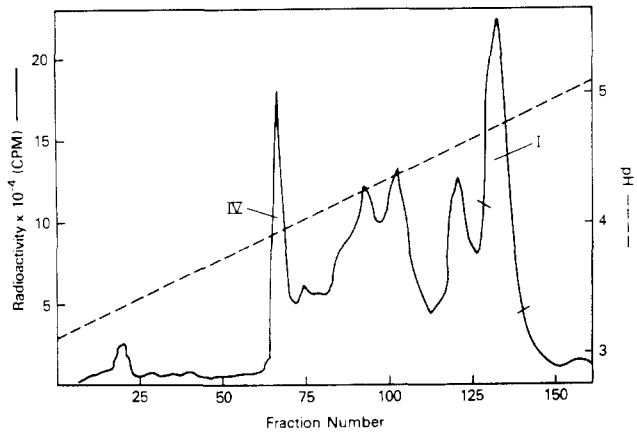

FIGURE 5: Elution of a mixture of peptides I, II, and IV (see Figure 4), after demaleylation, from a AG50W-X4 column (0.6 × 20 cm). Eluent, linear gradient 0.2 M acetic acid–pyridine (pH 3.1) to 2 M acetic acid–pyridine (pH 5.1) (molarity based on pyridine); temperature, 50°; fraction size, 0.35 ml. The roman numbers refer to peptides I and IV (see Results). The fractions between slash marks were pooled for further treatment.

shown by spectrophotometric determination (Butler et al., 1969). Subsequent tryptic digestion, therefore, caused fission only at the four Arg residues of the PA subunit, two of which are vicinal. Tryptic hydrolysis gave rise to the expected four peptides: residues 1–21 (I), 22–34 (II), 35–103 (III), and 105–127 (IV) in addition to undigested Dns-PA.

Gel filtration through Sephadex G-50 did not resolve peptides I, II, and IV, but separated this labeled peptide mixture from the unlabeled peptide III and from unhydrolyzed starting material representing one-third of the total radioactivity before tryptic digestion (Figure 4). Since peptide III is not labeled none of its five Lys residues is dansylated. This leaves only three Lys residues (9, 15, and 126) as possible points of attachment of the [^{3}H]Dns group. Further fractionation of the mixture of peptides I, II, and IV, after demaleylation, by chromatography on AG50W gave the elution profile shown in Figure 5. Amino acid analyses of the fractions under the various radioactive peaks, in conjunction with the distribution of the applied radioactivity among these peaks, revealed the following: the last two peaks (49% of the applied radioactivity) represent nearly pure peptide I. The amino acid analyses of the fractions

under these two peaks are virtually identical. The slight difference in mobility seems therefore to be due to a difference in the degree of demaleylation (see Discussion). The central double peak (43% of the applied radioactivity) represents peptide I contaminated with about 20% of peptide II or about 50% of peptide 1–34 (from incomplete tryptic hydrolysis at Arg-21). Phe and His served as reference amino acids in assigning the contamination to peptide II since these amino acids are present in peptide II in a 1:1 ratio, but absent in peptide I. The first peak (8% of the applied radioactivity) represents peptide IV contaminated with 15% of peptide I. Gly and Arg present in peptide I in a 3:1 ratio, but absent in peptide IV served as reference amino acids in assigning the contamination to peptide I. The entire radioactivity under the first peak is accounted for by the contamination with this peptide. Thus, Lys-126, which is in peptide IV, is not dansylated.

The labeled peptide I contains two Lys residues (9 and 15). In order to determine which one of these carries the label, the pooled fractions under the major radioactivity peak (peptide I) were treated with CNBr which caused partial fission of the peptide at Met-13. This was followed by one Edman degradation step, dansylation with unlabeled Dns-Cl, acid hydrolysis, and two-dimensional TLC. Autoradiography of the chromatogram revealed two labeled spots of N^{ϵ}-Dns-Lys and $N^{\alpha,\epsilon}$-bis-Dns-Lys with a radioactivity ratio of 4:1. Since Lys-9 is far removed from the N-terminal Gly and could therefore not have become available for N^{α}-dansylation after a single Edman degradation step, the bis-Dns-Lys is entirely derived from [^{3}H]Dns-Lys-15.

The N^{ϵ}-Dns-Lys in the acid hydrolysate of peptide I is also derived from [^{3}H]Dns-Lys-15. Its presence is due to incomplete fission of peptide I by CNBr and to incomplete Edman degradation and dansylation with unlabeled Dns-Cl. The following experiment confirms that it did not arise from a [^{3}H]Dns-labeled Lys-9 residue. [^{3}H]Dns-labeled PA was treated with CNBr. The reaction product consisting of a small N-terminal peptide (residues 1–13), a big C-terminal peptide (residues 14–127), and starting material was dialyzed. The diffusate containing the small peptide carried only a negligible amount of radioactivity (<5%). This very small amount is due to Dns-OH which was not completely removed from Dns-PA by extensive dialysis (Materials and Methods).

Not only peptide I, but also Dns-PA is split only incompletely by CNBr even in the presence of 6 *M* guanidine (Materials and Methods). This was shown by Edman degradation of the CNBr digest of Dns-PA, followed by dansylation of the reaction mixture with unlabeled Dns-Cl, acid hydrolysis, and two-dimensional TLC. As in the case of peptide I after a similar treatment, the only labeled Dns-amino acids obtained were N^{ϵ}-Dns-Lys and $N^{\alpha,\epsilon}$-bis-Dns-Lys, the former being derived from Dns-PA and the latter from the dansylated peptide 14–127. When the ethyl acetate extract of the reaction mixture obtained in the Edman degradation was submitted to gas chromatographic analysis (Pisano et al., 1972), the extracted phenylthiohydantoins were, as expected, those of Gly and of Val in a ratio of about 4:1.

Discussion

After it had been found that ANS binds to the same two sites on PA as T_4, it was logical to suspect that the chemically closely related Dns group would behave similarly. If this were so, Dns-Cl which contains, in contrast to ANS, a reactive sulfonyl chloride group capable of establishing a covalent bond with a nearby nucleophile could be used as an affinity labeling reagent for localizing the T_4 binding domain in PA. In order to determine whether the Dns group indeed seeks out the same sites as T_4 and ANS, spectrophotometric titrations as well as competitive equilibrium dialysis experiments were carried out with T_4 and Dns-Gly. The latter differs from Dns-Cl only by the replacement of the reactive sulfonyl chloride group with the unreactive sulfonylglycine group. The data that were obtained show that Dns competes with T_4 for the same sites on PA. Dns-Cl is therefore a suitable affinity labeling reagent. It has the advantage over other possible reagents that the Dns group can be easily detected in column effluents and on chromatograms by its strong fluorescence.

When PA was treated with 2.1 mol of Dns-Cl/mol of protein, 80% of the label could not be removed by treatment with charcoal and extensive dialysis against 6 *M* acid guanidine. Subsequent acid hydrolysis showed that Dns had attached itself to Lys. The presence of some Dns-OH in the acid hydrolysate can be explained either by the presence in the binding sites of noncovalently bound Dns-OH, formed by hydrolysis of Dns-Cl, or by the covalent attachment of Dns to certain other amino acids besides Lys. Dns-Cys and Dns-His would, if present, not have survived acid hydrolysis and would have yielded Dns-OH. By the same token it is certain that Dns-Cl has not reacted with Tyr since Dns-*O*-Tyr is resistant to acid hydrolysis, but was not present in the hydrolysate. Since only a small fraction of the radioactivity in the acid hydrolysate (15–20%) is due to [^{3}H]Dns-OH, Lys is the principle, if not only, amino acid to which Dns-Cl had attached itself.

In order to facilitate tryptic digestion of Dns-PA and to obtain only a few tryptic peptides, the Lys residues were protected by maleylation. The only drawback of this method of protection, which was also used by Kanda et al. (1974) for the same purpose, is that subsequent removal of the blocking group does not seem to be complete under the experimental conditions used. This is presumably the reason why all peptides obtained in the cation exchange chromatography were contaminated with some peptide I, the peptide which contains the labeled Lys-15 residue. Molecules of peptide I, which still contain more or less maleic acid residues, will migrate faster in a cation exchange column than completely deblocked peptide molecules.

The only amino acid residue in PA which was found to carry covalently bound Dns is Lys-15. This residue is located inside the central channel of PA and in the general area where T_4 binds according to the X-ray diffraction studies of Blake et al. (Blake et al., 1974). Only two of the four Lys-15 residues in PA bind Dns which is in agreement with the observation that only two molecules of Dns-Gly are bound noncovalently by PA. The same holds true for ANS and for T_4.

Acknowledgments

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Polysaccharide Intermediates Formed during Intracellular Transport of a Carbohydrate-Containing, Secreted Immunoglobulin Light Chain†

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ABSTRACT: Intracellular and secreted forms of the carbohydrate-containing light chain synthesized by MOPC-46 murine myeloma are heterogeneous in their single, carbohydrate moiety. To determine the number of different polysaccharide species contained in these forms of light chain, a technique was developed to separate and qualitatively analyze glycopeptides differing in carbohydrate composition. The glycopeptides were prepared by protease digestion of serologically precipitated, sugar radiolabeled light chain. Separation of glycopeptides was accomplished by column chromatography on Bio-Gel P6 polyacrylamide and paper electrophoresis. The carbohydrate moiety of secreted light chain contains *N*-acetylglucosamine, mannose, galactose, fucose, and variable amounts of *N*-glycolylneuraminic acid. Glycopeptides from secreted light chain were resolved into three species, differing in their content of *N*-glycolylneuraminic acid (0, 1, or 2 residues). Intracellular light chain glycopeptides were resolved into four species: the major glycopeptide species contained only "core" sugars, *N*-acetylglucosamine and mannose; another glycopeptide species contained core sugars and galactose; two glycopeptide species contained core sugars, galactose and one or two residues of *N*-glycolylneuraminic acid. Glycopeptides of intracellular light chain contained too few residues of fucose to be detected by incorporation of radioactive fucose. These findings corroborate the previous conclusion that carbohydrate attachment occurs in several steps to molecules destined to be secreted. Since a significant pool of light chain with core sugars, galactose, and neuraminic acid was found inside cells, attachment of fucose can now be designated as the final step in carbohydrate assembly, occurring close to or at the time of light chain secretion.

The light chain secreted by MOPC-46 myeloma cells possesses a polysaccharide moiety attached covalently to a single amino acid residue (Melchers, 1969). Previous studies have established certain features of the intracellular pathway leading to light chain secretion, and of the concomitant addition of sugar residues to the intracellular protein. The light chain polypeptide is synthesized on membrane-bound polyribosomes (Cioli and Lennox, 1973b). Pulse-chase kinetic studies, using radiolabeled leucine, show that light chain resides initially in the rough membrane fraction, is transported to the smooth membrane fraction, and is then secreted (Choi et al., 1971b; Melchers, 1971). The carbohydrate composition of secreted light chain (isolated from urine of tumor-bearing mice) is: 3 GlcNAc,[1] 4 Man, 4 Gal, 2 Fuc, and 0, 1, or 2 NGNA (Melchers, 1969). The polysaccharide composition of light chain isolated from either the rough membrane or smooth membrane fractions shows a deficiency in Gal and Fuc; NGNA was not analyzed (Choi et al., 1971b; Melchers, 1971). It was concluded that addition of sugar residues to light chain occurs in stages during the intracellular transport phase of secretion;

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[1] Abbreviations used are: GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Gal, D-galactose; Fuc, L-fucose; NGNA, *N*-glycolylneuraminic acid; NANA, *N*-acetylneuraminic acid; NP40, Nonidet P.40 (Shell Chem. Co., London, U.K.); RM, rough membrane; SM, smooth membrane.