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Affinity Labeling of Human Serum Prealbumin with *N*-Bromoacetyl-L-thyroxine

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SHEUE-YANN CHENG, MEIR WILCHEK,* HANS J. CAHNMANN, AND JACOB ROBBINS

From the Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Affinity labeling of human serum prealbumin with *N*-bromoacetyl-L-thyroxine ($BrAcT_4$) was used to investigate the binding domain for L-thyroxine (T_4) on prealbumin. Fluorescence titration with 8-anilinonaphthalene-1-sulfonate revealed a strong and a weak binding site for $BrAcT_4$ ($K_1 = 1 \times 10^8$ M^{-1}; $K_2 = 1 \times 10^6$ M^{-1}). The reaction of $BrAcT_4$ with prealbumin to form a covalent bond was inhibited in the presence of T_4 and binding of T_4 to prealbumin was nearly abolished after affinity labeling with $BrAcT_4$. Affinity labeling with 2 mol of $BrAcT_4$/mol of prealbumin resulted in covalent binding of 1 mol of ligand. Acid hydrolysis of affinity-labeled prealbumin gave N^ϵ-carboxymethyllysine and iminodiacetic acid, the latter being derived from the NH_2-terminal glycine. A combination of analytical procedures, including tryptic digestion after maleylation, cyanogen bromide cleavage, digestion with yeast protease C, and sequential Edman degradations, revealed that the N^ϵ-carboxymethyllysine was derived from lysine-9 and lysine-15 and that the affinity label had distributed itself among glycine-1, lysine-9, and lysine-15 in a ratio of 29:63:8.

Human serum prealbumin is involved in the transport of a hormone (thyroxine) and a vitamin (retinol). Whereas T_4[1] binds directly to prealbumin, retinol binds to another protein (retinol-binding protein) which in turn has a strong affinity for prealbumin. These ligands bind to prealbumin independently of each other (1). The two binding sites for T_4 are in a channel which runs through the center of the molecule and is formed by the tetrahedral arrangement of four identical subunits (2). The amino acid sequence of the subunit has been determined (3). Although the two sites for T_4 are identical, the second ligand molecule is bound much more weakly due to negative cooperativity (4). The precise area within the central channel to which T_4 attaches is not known.

We have previously reported affinity labeling of prealbumin with Dns-chloride (5). Although this labeling reagent can be used to determine the general area of the prealbumin molecule where T_4 binds, its structure is only remotely related to that of the hormone. We have, therefore, carried out affinity labeling experiments with $BrAcT_4$, which can react with nucleophilic groups in a protein to form T_4Ac-protein. The T_4Ac-residue differs from T_4 only by the replacement of one amino hydrogen with a carbonylmethyl ($COCH_2$—) residue.

After having ascertained that $BrAcT_4$ has a high affinity for prealbumin and that its binding to the protein is competitive with that of T_4, prealbumin was incubated with $BrAcT_4$ and the points of attachment of the ligand were determined in a series of degradation and fractionation procedures. It was found that $BrAcT_4$ had reacted mainly with lysine-9 and to a lesser extent with glycine-1 and with lysine-15.

MATERIALS AND METHODS

Prealbumin and trypsin were the same as those described by Cheng *et al.* (5). Yeast protease C (carboxypeptidase Y) was a gift from Dr. James F. Riordan. *N,N'*-Dicyclohexylcarbodiimide (Aldrich) was purified by distillation and *N*-hydroxysuccinimide (Aldrich) by recrystallization from ethyl acetate. Bromo[2-^{14}C]acetic acid (lyophilized powder, 27 Ci/mol) was purchased from Amersham/Searle. Dioxane and dimethylformamide were purified by distillation and maleic anhydride by sublimation. Guanidinium chloride was the ultrapure grade of Schwarz/Mann. Ans was the three times recrystallized magnesium salt. A molar extinction of 6240 M^{-1} cm^{-1} at 351 nm was used (6). T_4 was checked for purity and ^{125}I-T_4 was freed of contaminating iodide as described by Ferguson *et al.* (4). AcT_4 was synthesized from T_4 (sodium salt) and acetic anhydride in dimethylformamide and purified by crystallization from acetone:water. Ac-(3',5'-^{125}I)-T_4 was prepared by exchange labeling. Na^{125}I (250 μCi) and I_2 (50 μl of a 1.5% solution in methanol) were added to a solution of 2.8 mg of AcT_4 in 100 μl of methanol:water:acetic acid (95:5:1). After 15 min at room temperature, the solution was decolorized with a few drops of aqueous SO_2. Autoradiography, after purification by thin layer chromatography (silica gel, 5% acetic acid in acetone), showed a single spot which was eluted with dioxane:methanol (1:1). Iminodiacetic acid was purchased from Aldrich. Imino[2-^{14}C]diacetic acid was prepared by incubating 37 μg of iodo[2-^{14}C]acetic acid (Amersham/Searle) with 5 mg (65 μmol) of glycine in 3.3 M Na_2CO_3 (pH 10.4) for 1 h at 80°. After the addition of 12 mg (65 μmol) of unlabeled iodoacetic acid, the mixture was heated for another 3 h at 80°. Aliquots of the solution were used for electrophoresis. N^ϵ-[2-^{14}C]Carboxymethyllysine was prepared by heating 20 mg of polylysine·HBr (Sigma), M_r = 15,000 to 30,000 (96 μmol of lysine residues) with 28 mg (150 μmol) of iodoacetic acid and 0.1 to 0.2 μCi of iodo[2-^{14}C]acetic acid in 200 μl of 1 M Na_2CO_3 at 70° for 2 h. The reaction mixture was dialyzed, the dialysate lyophilized, and the residue hydrolyzed in 6 M HCl at 105° for 20 h. Aliquots of the hydrolysate were used for electrophoresis.

The methods used for equilibrium dialysis and for amino acid analysis have been described previously (5). Fluorescence titrations[2]

* Present address, Weizmann Institute of Science, Rehovot, Israel.

[1] The abbreviations used are: T_4, L-thyroxine; AcT_4, *N*-acetyl-L-thyroxine; $BrAcT_4$, *N*-bromoacetyl-L-thyroxine; T_4Ac-, L-thyroxyl-*N*-acetyl; Cm, carboxymethyl; Dns, dansyl (5-dimethylaminonaphthalene-1-sulfonyl); Ans, 8-anilinonaphthalene-1-sulfonate; CNBr, cyanogen bromide.

[2] S.-Y. Cheng, R. A. Pages, H. A. Saroff, H. Edelhoch, and J. Robbins, (1977) *Biochemistry* **16**, in press.

were carried out at 25° with a Turner spectrofluorometer, model 210. Excitation and emission wavelengths were set at 410 and 480 nm, respectively. Small increments of 2.16 μM $BrAcT_4$ in 0.1 M NaCl, 50 mM sodium phosphate (pH 7.4) were added to a solution containing 0.75 μM prealbumin and 169 μM Ans in the same buffer. The observed changes in fluorescence were corrected for fluctuations in lamp intensity and in photomultiplier response, which were checked with 37.5 μM prealbumin in 169 μM Ans using the same buffer. High voltage paper electrophoresis at pH 3.6 in pyridine:acetic acid:water (1:10:289) was carried out according to Katz *et al.* (7). A Beckman sequenator was used for automated Edman degradations. Manual Edman degradations were performed according to Sauer *et al.* (8) and end group determinations using Dns-chloride according to Gray (9). Two-dimensional thin layer chromatography (polyamide sheets) was used for the separation and identification of dansylated amino acids (10).

Synthesis of Br[2-^{14}C]AcT_4

A solution of *N*-hydroxysuccinimide (9 μmol) and *N,N'*-dicyclohexylcarbodiimide (11 μmol) in 40 μl of dimethylformamide was added to 9 μmol of bromo[2-^{14}C]acetic acid. The reaction mixture was kept, with occasional swirling, at room temperature for 45 min. After removal of dicyclohexylurea by centrifugation, the supernatant was added to 8 μmol of T_4 (sodium salt). After 1½ h, the solution was applied to two prewashed silica gel plates (Quantum Industries, Fairfield, N. J., Q1-F, 20 × 20 cm) for purification with the solvent ethyl acetate:acetic acid (9:1). The band with an $R_F \sim 0.4$, corresponding to $BrAcT_4$, was scraped off and eluted with dioxane (freshly redistilled over Drierite). The solvent was evaporated at <30° and the residue used for affinity labeling.

Synthesis of Unlabeled $BrAcT_4$

Unlabeled $BrAcT_4$ was prepared as a reference substance. Two synthetic methods were used.

Method A – A solution of *N,N'*-dicyclohexylcarbodiimide (200 μmol) in 400 μl of dioxane (freshly redistilled over Drierite) was added to bromoacetic acid (200 μmol) and hydroxysuccinimide (200 μmol). Thorough mixing resulted in dissolution of the solids, followed immediately by precipitation of dicyclohexylurea which was removed after 30 min by filtration and washed with 1.5 ml of dimethylformamide. Solid T_4 (sodium salt, 200 μmol) was dissolved in the combined filtrate and washing. After 20 min, the solution was poured into a 20% (w/v) aqueous solution of citric acid. Extraction with ethyl acetate, followed by washing with a saturated aqueous solution of NaCl, drying over $MgSO_4$, and concentration at <30° to a small volume gave slightly impure $BrAcT_4$. Final purification was done by preparative thin layer chromatography (PLQF plates) with the solvent system described above.

Method B – This method is based on that used by Matsuura and Cahnmann (11) for the synthesis of chloroacetyl-T_4. Solid T_4 (free acid, 200 μmol) was added to a 5- to 10-fold molar excess of bromoacetylbromide in 10 ml of dry ethyl acetate. The mixture was refluxed with a slow stream of dry N_2 passing through it until a clear solution was obtained (45 to 60 min). The solution was then concentrated to a small volume at <30°. After removal of a small amount of precipitate by centrifugation, the supernatant was purified by preparative thin layer chromatography as above.

Evaporation to dryness of solutions of $BrAcT_4$ in ethyl acetate was avoided, since the dry residue thus obtained was no longer completely soluble in ethyl acetate.

Affinity Labeling of Prealbumin with Br[2-^{14}C]AcT_4

Br[2-^{14}C]AcT_4 (57 Ci/mol, 74 nmol) in 0.1 ml of dioxane was added to a solution of 2 mg (37 nmol) of prealbumin in 1.9 ml of 0.1 M $NaHCO_3$ buffer, pH 8.6 (mole ratio of $BrAcT_4$/prealbumin = 2). After standing at room temperature for 24 h, the solution was dialyzed at room temperature against 0.37 mM 3,5,3',5'-tetraiodothyropropionic acid, a potent competitive inhibitor of binding of T_4 to prealbumin, in 0.1 M NaCl, 50 mM sodium phosphate (pH 7.9) for 24 h in order to displace noncovalently linked ligand. Removal of noncovalently linked $BrAcT_4$ and its hydrolysis products was virtually complete within 1 h. Salt and tetraiodothyropropionic acid were removed by further dialysis against deionized water. The retentate (T_4Ac-prealbumin) was then lyophilized.

Tryptic Digestion of Maleylated T_4[2-^{14}C]Ac-Prealbumin

A solution of 19.5 mg of T_4[2-^{14}C]Ac-prealbumin in 2 ml of 6 M guanidinium chloride, 50 mM sodium phosphate (pH 5.5) was kept at room temperature for 48 h. The pH was then adjusted with 5 M NaOH to 8.8 and 40 mg of maleic anhydride was added in small portions to the stirred solution. The pH was kept between 8 and 9 by the controlled addition of NaOH. After all the anhydride had been added, stirring was continued for another 30 min. The solution was then dialyzed against deionized water and lyophilized. The dry residue of maleylated T_4Ac-prealbumin was dissolved in 2 ml of 0.1 M NH_4HCO_3 buffer (pH 8.5) and treated with trypsin (substrate/enzyme weight ratio, 100:1) at 37° for 2 h. The tryptic digest was applied to a column of Sephadex G-50 (fine) (1.6 × 200 cm) and the peptides were eluted at room temperature with 0.1 M NH_4HCO_3 (pH 8.5).

Digestion with Yeast Protease C

A. Maleylated T_4[2-^{14}C]Ac-Prealbumin – A solution of 1 mg of maleylated T_4[2-^{14}C]Ac-prealbumin (see above) in 25 μl of 50 mM sodium citrate buffer (pH 5.5) was treated with yeast protease C at 37° for 4 h (substrate/enzyme weight ratio, 20:1). The reaction was stopped by immersion in boiling water for 2 min and the liberated amino acids were separated by high voltage paper electrophoresis. Liberated lysine was assayed spectrophotometrically (12).

B. Tryptic Peptides of Maleylated T_4[2-^{14}C]Ac-Prealbumin – A labeled mixture of peptides (~50 nmol of each peptide) obtained in the above mentioned gel filtration was dissolved in 400 μl of formic acid. After addition of 5 μl of 30% H_2O_2, the solution was incubated at 2° for 2 h, then lyophilized. The residue was taken up in water and the solution again lyophilized. The dissolution-lyophilization cycle was repeated twice more to remove all performic acid and then 40 μl of 50 mM sodium citrate (pH 5.5) and yeast protease C (substrate/enzyme weight ratio, 20:1) were added. Enzymic digestion and high voltage paper electrophoresis were carried out as described above. The radioactive spots, determined by autoradiography, were cut out and the radioactivity quantitated by combustion in a Packard Oxidizer and counting of $^{14}CO_2$ in Hyamine.

RESULTS

Binding of AcT_4 and $BrAcT_4$ to Prealbumin – Equilibrium dialysis showed that prealbumin has more than one binding site for AcT_4 (Fig. 1). The experimental data fit the theoretical curve for the two-independent-sites model of Ferguson *et al.* (4) up to 0.8 mol of AcT_4 bound. The association constant K_1 determined with this model is 1×10^7 M^{-1} which is one order of magnitude less than the K_1 found for T_4. K_2 could not be determined because of low solubility of AcT_4.

Both K_1 and K_2 could be determined by fluorescence titration of prealbumin-bound Ans with AcT_4. Using the two-independent-sites model, the values for K_1 and K_2 are 1×10^7 M^{-1} and 2×10^6 M^{-1}, respectively.

The binding parameters of $BrAcT_4$ cannot be determined by

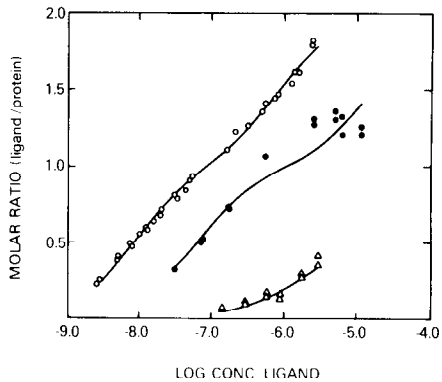

FIG. 1. Binding of (3',5'-^{125}I)-T_4 (○) and Ac-(3',5'-^{125}I)-T_4 (●) to prealbumin and of (3',5'-^{125}I)-T_4 to T_4Ac-prealbumin (△) as determined by equilibrium dialysis. The mole ratio of ligand/prealbumin is plotted against log free ligand. The *points* are experimental data. The *lines* are the theoretical curves (*cf.* Equation 1 of Ferguson *et al.* (4)). Prealbumin concentration, 0.50 to 0.94 μM (○) and 0.88 μM (●); T_4Ac-prealbumin concentration (1.2 T_4Ac- residues/mol of prealbumin), 0.89 μM (△).

equilibrium dialysis since the ligand reacts with prealbumin to form a covalent bond. However, the complete fluorescence titration of prealbumin-bound Ans with $BrAcT_4$ can be carried out within 15 to 20 min, whereas the establishment of a covalent bond between $BrAcT_4$ and prealbumin is a relatively slow process. Under the experimental conditions used for the affinity labeling of prealbumin ($BrAcT_4$ and prealbumin concentrations one order of magnitude higher than those used in fluorescence titration) only 0.24 mol and 0.48 mol of $BrAcT_4$ had formed a covalent bond with 1 mol of prealbumin within 2 h and 4 h, respectively. The binding constants obtained by fluorescence titration seem, therefore, to be reasonably accurate. The experimental values for K_1 and K_2 calculated from the two-independent-sites model are 1×10^8 M^{-1} and 1×10^6 M^{-1}, respectively.

Affinity Labeling of Prealbumin with Br[2-^{14}C]AcT$_4$ – In preliminary experiments, mole ratios of $BrAcT_4$ to prealbumin of 2, 4, and 12 resulted in the covalent attachment of 1.0, 1.7, and 3.1 mol of T_4Ac-/mol of prealbumin, respectively. Since the maximum specific binding should be 2.0, it is apparent that nonspecific covalent binding occurred at the highest ratio. Therefore, all subsequent experiments were done with $BrAcT_4$/prealbumin = 2. Evidence of labeling at the active site was obtained by comparing covalent binding of labeled T_4Ac- to prealbumin in the absence and in the presence of unlabeled T_4. In the absence of T_4, covalent binding was complete after 20 h and reached 1.0 mol of T_4Ac- bound/mol of prealbumin. In the presence of a 10-fold molar excess of unlabeled T_4 over labeled $BrAcT_4$, only 0.3 mol of T_4Ac- was bound/mol of prealbumin. Further evidence was obtained by studying the binding of T_4 to T_4Ac-prealbumin (mole ratio of T_4Ac/prealbumin = 1.2) by equilibrium dialysis. As shown in Fig. 1, binding of T_4 to the modified protein was nearly abolished.

Determination of Site of Attachment of T_4Ac- to Prealbumin – Acid hydrolysis of T_4[2-^{14}C]Ac-prealbumin, followed by high voltage paper electrophoresis, gave two radioactive spots, with a radioactivity ratio of 29:71 (Table I), which accounted for 96% of the radioactivity. Comparison with authentic samples indicated that the less radioactive spot, which moved toward the anode (mobility, 1.6 × glycolic acid), was iminodiacetic acid (derived from glycine-1) and that the more radioactive spot, which moved toward the cathode (mobility, 0.13 × lysine), was N^{ϵ}-Cm-lysine. Their identity was confirmed by amino acid analysis. In this procedure, the labeled compounds coeluted with authentic N^{ϵ}-Cm-lysine immediately after methionine, and with authentic iminodiacetic acid shortly after cysteic acid. The iminodiacetic acid/Cm-lysine ratio determined in 10 separate experiments was 0.40 ± 0.04 (mean ± S.D.). An additional minor component, which contained only 1.5% of the total radioactivity, was identified by high voltage electrophoresis and by ion exchange chromatography as glycolic acid.

When affinity labeling was carried out in the presence of an excess of T_4, the acid hydrolysate of T_4[2-^{14}C]Ac-prealbumin showed a decrease of both of the major labeled products (Table I). Iminodiacetic acid was reduced 2-fold and Cm-lysine was reduced 4-fold. This indicates that both glycine-1 and lysine were specifically labeled by $BrAcT_4$.

There is a total of 8 lysine residues per subunit of prealbumin. In order to determine which lysines had been labeled, T_4Ac-prealbumin was treated with trypsin after blocking of all free ϵ-amino groups by maleylation in the presence of 6 M guanidinium chloride. Since 2 of the 4 arginine residues per subunit of prealbumin are vicinal, 4 peptides are expected from the known sequence of prealbumin (3), *viz.* residues 1 to 21 (Peptide I), 22 to 34 (Peptide II), 35 to 103 (Peptide III), and 105 to 127 (Peptide IV). Fig. 2 is a representation of the prealbumin subunit, in which only the residues important to the present work are indicated. When the peptide mixture in the tryptic hydrolysate was chromatographed on Sephadex G-50, the elution profile shown in Fig. 3 was obtained. Amino acid analysis and end group determination of pooled fractions (Table II) showed that the excluded radioactive peak was undigested T_4Ac-prealbumin and that the slightly retarded nonradioactive peak was Peptide III. Since Peptide III was not labeled, 5 lysine residues (Fig. 2) are eliminated as possible attachment sites.

In six experiments, undigested T_4Ac-prealbumin accounted for 20 to 25% of the total radioactivity. The strongly retarded radioactive peak (Fractions 70 to 94 in Fig. 3) contained 93 to 98% of the hydrolyzed radioactivity. Amino acid analysis of this pool indicated that it contained a mixture of Peptides I, II, and IV, and end group analysis showed that Dns-glycine (from Peptides I and II) and *N*,*O*-bis-Dns-tyrosine (from Peptide IV) were the only NH_2-terminal amino acids. This finding is consistent with earlier work (3, 5). This peptide mixture coincided with the major radioactive peak containing three different lysine residues, *i.e.* lysine-9, lysine-15, and lysine-126 (*cf.* Fig. 2). Lysine-126 is the 2nd amino acid residue from the COOH terminus of prealbumin. Treatment of T_4[2-^{14}C]Ac-prealbumin with yeast protease C resulted in the removal of several amino acids including the quantitative removal of lysine-126. Autoradiography of the electrophoretogram of the enzymic digest showed that this lysine was not labeled. This leaves only the 2 lysine residues of Peptide I (lysine-9 and -15) that could have been labeled. Therefore, separation of Peptide I from Peptides II and IV was not required for further analysis.

Sequential Edman degradation of unfractionated T_4[2-^{14}C]Ac-prealbumin (20 steps) showed release of 20% of the total

TABLE I

Affinity labeling of prealbumin with bromo[2-^{14}C]acetylthyroxine in absence and in presence of thyroxine

Prealbumin concentration was 18.5 μM; mole ratio of bromo[2-^{14}C]acetylthyroxine/prealbumin, 2; mole ratio of thyroxine/bromo[12-^{14}C]acetylthyroxine, 10.

Labeled species[a]		Thyroxine absent[b]	Thyroxine present
Before hydrolysis	After hydrolysis		
		mol/mol prealbumin	
Thyroxyl-*N*-acetylglycine-1	Iminodiacetic acid	0.29 ± 0.02	0.15
N^{ϵ}-(Thyroxyl-*N*-acetyl)lysine	N^{ϵ}-Cm-lysine	0.71 ± 0.02	0.18

[a] Determined by hydrolysis with 6 M HCl, followed by high voltage paper electrophoresis.

[b] Mean ± S.D. from 10 experiments.

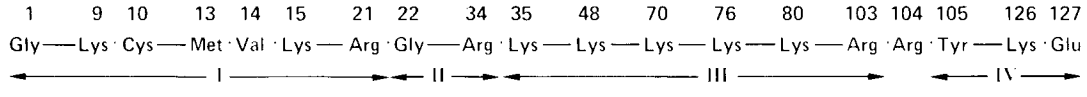

FIG. 2. Primary structure of the prealbumin subunit (from Kanda *et al.* (3)). Only pertinent amino acid residues and tryptic cleavage points are shown. *Roman numerals* denote tryptic peptides of maleylated prealbumin.

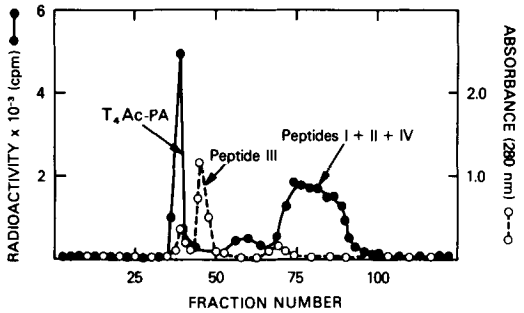

FIG. 3. Elution of the tryptic digest of maleylated T_4[2-^{14}C]Ac-prealbumin (T_4Ac-PA) from a column of Sephadex G-50 (fine) (1.6 × 200 cm). Eluent, 0.1 M NH_4HCO_3 (pH 8.5); temperature, 25°; flow rate, 4.7 ml/h; fraction size, 4 ml. The fractions between *slash marks* were pooled for treatment with yeast protease C. *Roman numerals* denote Peptides I through IV (*cf.* Fig. 2).

TABLE II

Amino acid analysis of gel filtration peaks

Amino acid	Tryptic digestion of T_4Ac-prealbumin				CNBr cleavage of T_4Ac-prealbumin	
	Undigested prealbumin		Peptide III		Peptide 1-13	
	Kanda *et al.* (3)	Present work[a]	Kanda *et al.* (3)	Present work[b]	Kanda *et al.* (3)	Present work[c]
Lysine	8	7.5	5	4.6	1	0.7
Histidine	4	3.9	3	2.5		
Arginine	4	3.8	1	1.1		
Aspartic acid	8	8.7	5	4.7		
Threonine	12	11.2	6	5.8	2	1.7
Serine	11	10.5	6	5.8	1	0.9
Glutamic acid	12	11.9	10	9.6	1	1.0
Proline	8	8.1	3	3.4	2	1.7
Glycine	10	10.4	6	6.3	3	2.7
Alanine	12	12.3	6	6.1		
Cysteine	1	ND[d]			1	ND[d]
Valine	11	11.4	4	3.5		
Methionine	1	0.9			1	0.7[e]
Isoleucine	5	4.7	3	2.8		
Leucine	7	6.2	3	2.7	1	0.9
Tyrosine	5	4.3	2	1.7		
Phenylalanine	5	5.0	4	4.8		
Danysation products	Dns-Gly, N^{ϵ}-Dns-Lys		Dns-$N^{\alpha,\epsilon}$-bis-Lys, N^{ϵ}-Dns-Lys		Dns-Gly, N^{ϵ}-Dns-Lys	

[a] Unretarded radioactive peak in Fig. 3.
[b] Slightly retarded nonradioactive peak in Fig. 3.
[c] *Peak B* in Fig. 5. Since Lys-9 and Gly-1 are modified, the theoretical value should be <1 and <3, respectively.
[d] Not determined.
[e] Determined as homoserine.

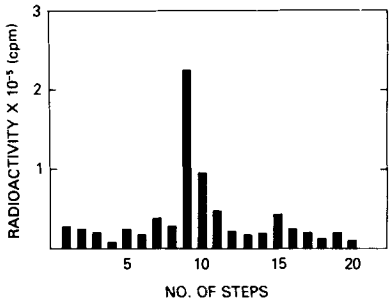

FIG. 4. Sequential Edman degradation (20 steps) of T_4[2-^{14}C]Ac-prealbumin (0.1 μmol, 1.7 μCi).

TABLE III

Distribution of T_4Ac in Prealbumin

Starting material		Method of cleavage	Distribution of label	
			Lys-15	Gly-1 + Lys-9
	dpm × 10^{-5}		*dpm* × 10^{-5}	
Peptide I + II + IV[a]	2.2	Protease C	0.17[b]	1.9[b]
T_4Ac-prealbumin	14	CNBr	0.38[c]	4.0[d]

[a] Retarded radioactive peak (Fig. 3) from tryptic digest of maleylated T_4Ac-prealbumin.
[b] Determined by high voltage electrophoresis.
[c] Radioactivity released from *Peak A* in Fig. 5 by sequential Edman degradation (4 steps).
[d] Total counts in *Peak B* of Fig. 5.

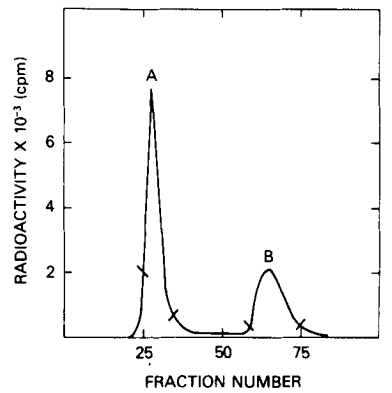

FIG. 5. Elution of the CNBr digest of T_4[2-^{14}C]Ac-prealbumin from a column of Sephadex G-50 (fine) (0.7 × 85 cm). Eluent, 10% acetic acid; temperature, 25°; flow rate, 4.5 ml/h; fraction size, 0.5 ml. The fractions between *slash marks* were pooled for amino acid analysis, end group determination, or Edman degradation. *Peak A* is a mixture of peptide 14-127 and uncleaved T_4Ac-prealbumin; *Peak B* is Peptide 1-13.

radioactivity at Steps 9 and 10 (Fig. 4), indicating definite labeling of lysine-9. The relatively low yield of radioactivity in the Edman degradation is due to several factors. As seen in Fig. 4, substitution of a hydrogen in the α-amino group of glycine-1 with the bulky T_4Ac- makes the amino terminus of T_4Ac-prealbumin resistant to cleavage. (As shown above, T_4Ac-glycine-1 contains 29% of the radioactivity in T_4Ac-prealbumin.) Furthermore, the NH_2-terminal sequence of prealbumin contains several amino acids that are known to result in poor cleavage yield; *i.e.* proline-2, threonine-3, threonine-5, and serine-8 (13). For these reasons, it was not possible to decide whether the radioactivity released at Step 15 came from T_4Ac-lysine-15 or simply represented background variation. Two experiments were therefore performed. Digestion of the mixture of Peptides I, II, and IV with yeast protease C and CNBr cleavage of T_4[2-^{14}C]Ac-prealbumin. For the enzymic digestion, the peptide mixture was first treated with performic acid in order to convert cysteine-10 to cysteic acid, which is easily recognizable in electrophoretograms. The experimental conditions for the treatment with protease C were chosen so that no free cysteic acid could be detected in the digest, thus proving that no lysine-9 had been released by the enzyme. High voltage electrophoresis of the digest revealed two radioactive spots with a radioactivity ratio 1:11 (Table III). The less radioactive spot which moved toward the cathode was identified as Cm-lysine by comparison with an authentic sample (electrophoresis and thin layer chromatography on polyamide sheets). The other spot which moved toward the anode was the

remaining undigested peptide (residues 1 through ~12 or 13). Since amino acid analysis showed that it contained all the cysteic acid and, therefore, also all the lysine-9, the liberated Cm-lysine could have arisen only from lysine-15.

Additional evidence for the attachment of some of the T_4[2-^{14}C]Ac-residues to lysine-15 was obtained by CNBr cleavage of labeled prealbumin. In order to make the protein more susceptible to attack by CNBr and to prevent air oxidation of methionine-13, T_4Ac-prealbumin was first treated with guanidinium chloride and mercaptoethanol and the reaction with CNBr was carried out in the presence of these agents. Gel filtration of the digest through Sephadex G-50 resulted in two well separated radioactive peaks (Fig. 5). Amino acid analysis and end group determination by dansylation (Table II) showed that the more retarded *Peak B* was the peptide consisting of residues 1 through 13. *Peak A*, eluted near the void volume, was a mixture of Peptide 14 through 127 and of uncleaved T_4Ac-prealbumin. Edman degradation of this mixture was followed by identification of the released phenylthiohydantoins by gas chromatography and by mass spectrometry. As expected, only the phenylthiohydantoins of valine-14 and of glycine-1 were observed after a single degradation step (*cf.* Fig. 2). Sequential Edman degradation (4 steps) of the combined fractions under *Peak A* released significant radioactivity in Steps 2 and 3, which could have arisen only from lysine-15 in Peptide 14–127. (No significant amount of glycine-1 radioactivity from uncleaved T_4Ac-prealbumin is released—see above.) The radioactivity of *Peak B* (Peptide 1–13) is due to that of glycine-1 and of lysine-9. The ratio of the radioactivity released by Edman degradation of *Peak A* to the total radioactivity in *Peak B* corresponds, therefore, to lysine-15/(glycine-1 + lysine-9). As shown in Table III, that ratio was again 1:11. Although cleavage by CNBr was incomplete (~35%), this does not affect the calculation of this radioactivity ratio.

From the 1:11 ratio of lysine-15/(glycine-1 + lysine-9) and the 29:71 ratio of glycine-1/(lysine-9 + lysine-15), the distribution of T_4Ac- among the three labeled amino acids can be calculated. The distribution ratio for glycine-1, lysine-9, and lysine-15 was 29:63:8.

DISCUSSION

The affinity of $BrAcT_4$ for prealbumin is nearly the same as that of T_4 and one order of magnitude greater than that of AcT_4. Furthermore, inhibition experiments indicate that T_4Ac- and T_4 bind to prealbumin at the same site. Therefore, $BrAcT_4$ is an excellent affinity labeling reagent for the T_4 binding site on the prealbumin molecule.

The formation of Cm-lysine upon acid hydrolysis of T_4Ac-prealbumin is more meaningful than the formation of Dns-lysine obtained from dansylated prealbumin (5). Dns-cysteine as well as Dns-histidine do not survive acid hydrolysis and would, therefore, not have been detected, whereas T_4Ac-cysteine and T_4Ac-histidine are converted to the corresponding Cm derivatives which would still carry the radioactive label.

T_4Ac-methionine would have given rise, upon acid hydrolysis, to labeled Cm-homocysteine, and to unlabeled homoserine or homoserine lactone (14), none of which were found. The absence of T_4Ac-methionine was confirmed by an experiment in which T_4Ac-prealbumin was treated with performic acid, which converts only unsubstituted methionine residues to methionine sulfone. This conversion was quantitative (1 mol of sulfone/mol of prealbumin) as shown by amino acid analysis. The trace amount of labeled glycolic acid (1.5% of the T_4Ac-radioactivity) found after acid hydrolysis of T_4Ac-prealbumin probably arose from traces of unbound $BrAcT_4$ and not from labeled aspartic or glutamic acid, since that label would have been largely removed by alkaline hydrolysis, followed by dialysis (15). No such removal was observed after exposure to 0.1 M NaOH for 1 h. $BrAcT_4$ has also not reacted with histidine, serine, threonine, cysteine, and tyrosine, since their T_4Ac-derivatives would have yielded, after acid hydrolysis, the corresponding Cm derivatives (14, 16). No tryptophan residues were labeled since the 2 tryptophan residues in prealbumin are in the unlabeled Peptide III. This peptide also contains 5 lysine residues.

The remaining 3 lysine residues in prealbumin are in Peptides I and IV. The one in Peptide IV (lysine-126) was shown to be unlabeled by the quantitative removal of unlabeled lysine-126 by treatment of labeled prealbumin with yeast protease C. Since the 2 other lysine residues (9 and 15) are in Peptide I, resolution of the mixture of Peptides I, II, and IV obtained from the tryptic digest of maleylated T_4Ac-prealbumin (Fig. 3) was not necessary. Sequential Edman degradation of T_4Ac-prealbumin (Fig. 4) showed that lysine-9 was labeled. Since the yield in sequential Edman degradations decreases with the number of steps, and since lysine-15 contained only 8% of the label in T_4Ac-prealbumin, it was not possible to establish the labeling of lysine-15 by this method. However, treatment of the labeled mixture of Peptides I, II, and IV with yeast protease C proved that lysine-15 was labeled. This was confirmed by treatment of T_4Ac-prealbumin with CNBr, followed by gel filtration and a 4-step Edman degradation.

Acid hydrolysis of T_4Ac-prealbumin, followed by high voltage electrophoresis showed that not only lysine, but also the NH_2-terminal glycine was labeled. The radioactivity in iminodiacetic acid (derived from glycine-1) and in N^{ϵ}-Cm-lysine (derived from lysine-9 and lysine-15) accounted for 96% of the total radioactivity, in a 29:71 ratio. The fact that no significant amount of radioactivity was released in Step 1 of the sequential Edman degradation of T_4Ac-prealbumin indicates that substitution of an α-amino group with the bulky T_4Ac- prevents normal Edman degradation. However, this does not interfere with detection of T_4Ac-lysine-9, since the dimensions of the prealbumin binding site (2) do not permit the introduction of more than 1 ligand molecule within the binding domain. Thus, any one subunit can contain the label only on a single residue; *i.e.* on glycine-1, lysine-9, or lysine-15.

The radioactivity ratio lysine-15/(glycine-1 + lysine-9) was 1:11, as determined by two independent methods: limited degradation of Peptide I by yeast protease C and CNBr cleavage of T_4Ac-prealbumin. From this, and from the 29:71 radioactivity ratio for glycine-1/(lysine-9 + lysine-15) determined by acid hydrolysis of T_4Ac-prealbumin, it was calculated that the affinity label had distributed itself among glycine-1, lysine-9, and lysine-15 in the ratio 29:63:8.

Lysine-15 is located inside the central channel of prealbumin, formed by four identical subunits, whereas lysine-9 is near the channel entrance (2). Glycine-1 is located outside the channel, but does not have a fixed position because the NH_2-terminal portion of prealbumin (residues 1 to 9) is flexible.[3] Dns-chloride, a more compact molecule than T_4 or $BrAcT_4$, was found to attach itself only to lysine-15 (5). The meaning of the attachment of $BrAcT_4$ to 2 different lysine residues and to the NH_2-terminal glycine is not clear. If one assumes that the binding site for T_4 and its analog $BrAcT_4$ is deep inside the channel (with the side chain near lysine-15), then the ligand must pass by lysine-9 in order to reach the binding site. A

[3] C. C. F. Blake, personal communication.

capture of $BrAcT_4$ by lysine-9 in the course of this migration, however, does not seem likely since the reaction of $BrAcT_4$ with prealbumin is slow.

Lysine-9 and lysine-15 may well lie within the same T_4Ac-binding domain, but it seems less likely, in view of our present knowledge of the molecular structure of prealbumin, that glycine-1 also lies within the same domain. When affinity labeling of prealbumin was carried out in the presence of a 10-fold molar excess of T_4 over $BrAcT_4$, there was a 2-fold decrease in the labeling of glycine-1. This suggests that glycine-1 may be within the T_4 binding domain. However, under the same conditions, T_4 interfered to a greater extent with the labeling of lysine-9 and lysine-15 than with that of glycine-1. This indicates that glycine-1 may also be labeled nonspecifically. The flexibility of the NH_2-terminal portion of prealbumin may explain the susceptibility of glycine-1 to interaction with the ligand, since glycine-1 could come so close to the channel entrance that a frequent encounter with $BrAcT_4$ molecules is ensured.

These considerations must remain speculative until a deeper insight into the topography of the binding site and the mechanism of T_4 binding has been gained through more chemical and x-ray diffraction studies.

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