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Identification of the Corticotropin Binding Domain of Bovine Serum Albumin by Photoaffinity Labeling†

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ABSTRACT: The interaction of the pituitary hormone corticotropin (ACTH) with bovine serum albumin (BSA) was investigated by photoaffinity labeling with 2-nitro-4-azidophenylsulfenyl (2,4-NAPS) derivatives of ACTH and [Trp(SH)^9]ACTH. Nearly 30 mol % of tritiated [2,4-NAPS-Trp^9]ACTH was covalently bound to BSA at a molar ratio of hormone:BSA of 1.33. The [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex was isolated, and the CNBr fragments of the complex were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The radioactivity was predominantly associated with the amino-terminal CNBr fragment corresponding to residues 1–183 in BSA. This result was confirmed by studies of the inhibition of covalent labeling of BSA by photoreactive ACTH. 8-Anilinonaphthalenesulfonic acid which binds to the amino-terminal domain of BSA strongly inhibited the photolabeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH. Palmitate and progesterone, known to bind to the carboxy-terminal domains of BSA, did not inhibit the incorporation of [2,4-NAPS-Trp^9][^{3}H]ACTH into BSA. The removal of ACTH from the covalent complexes was also investigated. The release of ACTH from the [2,4-NAPSS-Trp^9]ACTH–BSA complex by treatment with β-mercaptoethanol was complete in 6 h, but only 80% of ACTH was released from [2,4-NAPS-Trp^9]ACTH–BSA under these conditions.

We have prepared and characterized several photoreactive derivatives of the pituitary hormone corticotropin (ACTH)[1] by selective modification of the tryptophan residue of the hormone (Muramoto & Ramachandran, 1980, 1981). These derivatives were shown to attach covalently to a crude preparation of ovine pituitary ACTH binding protein upon photolysis. In order to further evaluate the utility of photoreactive ACTH derivatives as photoaffinity labels for ACTH receptors, we have investigated the covalent attachment of these derivatives to bovine serum albumin (BSA). It is known that several peptides including gastrin (Galardy et al., 1974), α-melanotropin (Medzhiradszky, 1976), and ACTH (Stouffer & Hsu, 1966; Ramachandran & Behrens, 1977) bind to BSA. The binding of ACTH and α-melanotropin to BSA has been studied by using equilibrium dialysis and gel filtration. Although some specific interactions between these peptides and BSA were revealed by these studies, the segment(s) of BSA involved in the binding is (are) not known. In this paper we describe the photoaffinity labeling of BSA by NAPS derivatives of ACTH and the identification of the binding domain of BSA for the hormone.

Materials and Methods

BSA was obtained from Miles Laboratories (Elkhart, IN) and purified as described (Ramachandran et al., 1972). The photoreactive derivatives [2,4-NAPS-Trp^9]ACTH and [2,5-NAPS-Trp^9]ACTH were prepared as described previously (Muramoto & Ramachandran, 1980). The preparation of the NAPS derivatives of [Trp(SH)^9]ACTH is presented in the preceding paper in this issue. [3,5-$^3H_2$-Tyr^{2,23}]ACTH ([^{3}H]ACTH) was prepared according to Ramachandran & Behrens (1977). S-Carbamidomethylated BSA was prepared according to King & Spencer (1970) and [Met(O)^4]ACTH by oxidation with hydrogen peroxide (Dedman et al., 1961).

8-Anilinonaphthalenesulfonic acid (Ans) was obtained from Eastman, palmitic acid from Matheson Coleman and Bell, indole from Fisher Scientific Co., progesterone from Stearaloids, and L-tryptophan from Schwarz/Mann.

Preparation of 2-Nitro-5-(acetylamino)phenylsulfenyl-L-tryptophan (Ac-NAmPS-Trp). To 1 g of 2-nitro-5-aminophenyl benzyl sulfide (Muramoto & Ramachandran, 1980) dissolved in 20 mL of acetic anhydride was added 2 drops of concentrated sulfuric acid as a catalyst. The product was concentrated after 10 min at room temperature and crystallized from ethyl acetate to give 0.93 g (81%), mp 153–154 °C, homogeneous on TLC on silica gel G, R_f 0.25 in benzene–acetic acid (10:1 v/v).

2-Nitro-5-(acetylamino)phenyl benzyl sulfide (600 mg) was reacted with sulfuryl chloride in dichloromethane to give 385 mg (78%) of 2-nitro-5-(acetylamino)phenylsulfenyl chloride. The product was used directly for the next reaction.

2-Nitro-5-(acetylamino)phenylsulfenyl chloride (340 mg) was added to 340 mg of L-tryptophan dissolved in 10 mL of glacial acetic acid. The reaction mixture was left for 24 h at room temperature in the dark and poured into 50 mL of

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[1] Abbreviations used: ACTH, corticotropin; BSA, bovine serum albumin; [2,4(5)-NAPS-Trp^9]ACTH, [2-nitro-4(5)-azidophenylsulfenyl-Trp^9]ACTH; Ans, 8-anilinonaphthalenesulfonic acid; Ac-NAmPS-Trp, 2-nitro-5-(acetylamino)phenylsulfenyl-L-tryptophan; [2,4(5)-NAPSS-Trp^9]ACTH, 2-nitro-4(5)-azidophenylsulfenyl derivative(s) of [Trp(SH)^9]ACTH; [Trp(SH)^9]ACTH, [9-(2-mercapto)tryptophan]-corticotropin; EDTA, ethylenediaminetetraacetic acid.

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ice-cold water. The precipitate was filtered, washed with water, and triturated with hot methanol to give 272 mg (48%) of 2-nitro-5-(acetylamino)phenylsulfenyl-L-tryptophan (Ac-NAmPS-Trp), mp 255–260 °C dec, homogeneous on TLC on silica gel, R_f 0.61 in 1-butanol–acetic acid–water (4:1:1 v/v/v). Anal. Calcd for $C_{19}H_{18}N_4O_5S \cdot 0.5H_2O$ (M_r 423.4): C, 53.89; H, 4.52; N, 13.23. Found: C, 53.41; H, 4.45; N, 13.05.

Photoaffinity Labeling of NAPS-ACTH Binding Sites on BSA. BSA (100 μg, 7.5 μM) and NAPS-ACTH (10 μg, 10 μM) containing NAPS-[^{3}H]ACTH (7×10^5 cpm) were incubated in 0.2 mL of 50 mM phosphate buffer (pH 7.4) for 3 h at 22 °C. For the experiment of the inhibition of the binding, inhibitors were added to the mixture before incubation. The mixture was photolyzed in a glass tube (12 × 75 mm) at 0 °C for the indicated times. A Blak-Ray UV lamp emitting principal radiation at 366 nm was used for photolysis at a distance of 10–15 cm from the test tube. After lyophilization, the photolyzed sample was dissolved in 0.2 mL of 6 M guanidine hydrochloride and subjected to gel filtration on a Sephadex G-75 column (0.8 × 28 cm) equilibrated with 6 M guanidine hydrochloride. Fractions of 0.4 mL were collected, and their radioactivity was measured in 4.5 mL of PCS (Amersham).

The covalent binding percentage of NAPS-ACTH with BSA was calculated by dividing the radioactivity eluted with the BSA fraction by the total radioactivity eluted from the column. Radioactivity bound to BSA in the unphotolyzed sample was subtracted from the values to obtain specifically bound radioactivity.

For the experiments of the thiolysis and reduction of photolyzed NAPS-[^{3}H]ACTH–BSA complex, we prepared the complex on a larger scale. NAPS-[^{3}H]ACTH (0.5 mg, 9×10^6 cpm) and 10 mg of BSA in 5 mL of 50 mM phosphate buffer (pH 7.4) were incubated for 3 h at 22 °C and photolyzed for 15 min at 0 °C. After photolysis, the NAPS-[^{3}H]ACTH–BSA complex was separated from NAPS-[^{3}H]-ACTH by gel filtration on a Sephadex G-75 column (1.3 × 30 cm) equilibrated with 6 M guanidine hydrochloride. The NAPS-[^{3}H]ACTH–BSA fraction was dialyzed against deionized water for 2 days and lyophilized.

Thiolysis and Reduction of Photolyzed NAPS-ACTH–BSA Complexes. One milligram of NAPS-[^{3}H]ACTH–BSA complex in a test tube (12 × 75 mm) was dissolved in 1 mL of degassed 0.1 M $NaHCO_3$ containing 2 mM EDTA and 2% β-mercaptoethanol. After being flushed with nitrogen, the tube was closed and kept at 37 °C. The reaction was terminated by lyophilization after the addition of 1 mg of cysteine in 0.1 mL of distilled water. The residue was dissolved in 0.2 mL of 6 M guanidine hydrochloride and chromatographed on Sephadex G-75 (0.8 × 28 cm) equilibrated with 6 M guanidine hydrochloride.

Cyanogen Bromide Cleavage of Photolyzed NAPS-ACTH–BSA Complex. The photolyzed [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex, which was prepared in the same way as described above, was cleaved with cyanogen bromide in formic acid according to King & Spencer (1970). The lyophilized fragments were dissolved in 1% sodium dodecyl sulfate ($NaDodSO_4$) and subjected to electrophoresis on 15% polyacrylamide–$NaDodSO_4$ slab gel (1.5-mm thickness) according to Laemmli (1970). The gel was sliced, digested with 0.5 mL of NCS solubilizer (Amersham)/water (9:1 v/v) at 37 °C for 24 h, and incubated at room temperature for 24 h after mixing with PCS for counting. Radioactive peaks were identified by comparing Coomassie blue stained bands of NAPS-ACTH–BSA complex and BSA fragments.

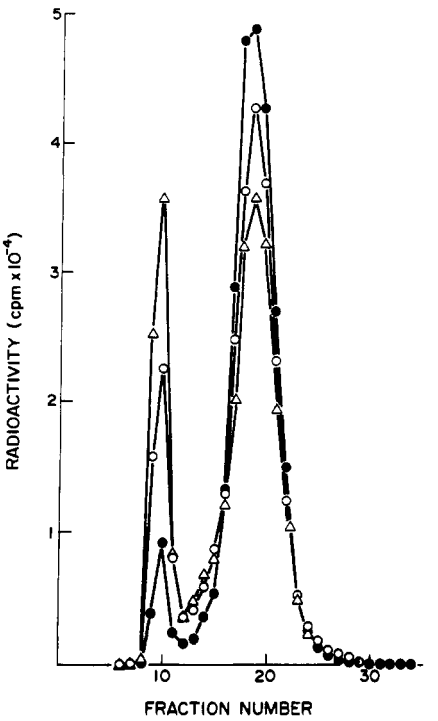

FIGURE 1: Separation of the covalent complex of [2,4-NAPS-Trp^9][^{3}H]ACTH and BSA from free [2,4-NAPS-Trp^9][^{3}H]ACTH. The photoreactive derivative (10 μM, 7×10^5 cpm) was incubated with BSA (7.5 μM) in 0.2 mL of 50 mM phosphate buffer, pH 7.4, and photolyzed at 0 °C for 0 (●), 5 (○) and 60 min (△). The photolysis mixture was lyophilized, redissolved in 0.2 mL of 6 M guanidine hydrochloride, and fractionated on Sephadex G-75 (0.8 × 28 cm) equilibrated with 6 M guanidine hydrochloride. Flow rate, 6 mL/h; fractions, 0.4 mL.

Binding of [^{3}H]ACTH to BSA. BSA (100 μg, 7.5 μM) and [^{3}H]ACTH (10 μg, 6×10^5 cpm) were incubated in 0.2-mL total volume of 40 mM ammonium acetate buffer, pH 5, for 48 h at 4 °C. [^{3}H]ACTH bound to BSA was separated from free [^{3}H]ACTH by gel filtration on a Sephadex G-75 column (0.8 × 29 cm) equilibrated with 0.1 M ammonium acetate, pH 5, at 4 °C. The column was treated with 1 mg of ACTH before use to prevent adsorption of [^{3}H]ACTH. Fractions (0.4 mL) were mixed with PCS scintillant and counted.

Results

Photoaffinity Labeling of BSA. When BSA was incubated with 1.33 molar equiv of [2,4-NAPS-Trp^9][^{3}H]ACTH at pH 7.4 and photolyzed, a significant amount of the hormone was covalently attached to BSA as shown in Figure 1. Since the photolysis mixture was separated by gel filtration in the presence of 6 M guanidine hydrochloride, the radioactivity emerging in the excluded volume with BSA represents [^{3}H]-ACTH attached covalently to BSA. It is evident that the incorporation of radioactivity into BSA increased with time of photolysis. The kinetics of photoaffinity labeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH and [2,5-NAPS-Trp^9][^{3}H]-ACTH were investigated. Both derivatives caused maximal labeling of BSA after 30-min photolysis. However, the amount of [2,4-NAPS-Trp^9][^{3}H]ACTH incorporated into BSA was more than twice the amount of [2,5-NAPS-Trp^9][^{3}H]ACTH bound. The photoaffinity labeling of BSA by several photoreactive derivatives of ACTH is compared in Table I. At a molar ratio for NAPS-[^{3}H]ACTH–BSA of 0.66, the 2,4-NAPS derivatives of ACTH and [Trp(SH)^9]ACTH were both incorporated to a greater extent compared to the corresponding 2,5-NAPS derivatives. This result agrees with the differences in photoreactivities of 2,4-NAPS groups and 2,5-NAPS groups

Table I: Covalent Attachment of ACTH to BSA by Photoaffinity Labeling

ACTH derivative	mol % ACTH derivative bound to BSA[a] A	B	C
[2,4-NAPS-Trp^9][^{3}H]ACTH	18	26	28
[2,4-NAPSS-Trp^9][^{3}H]ACTH	17		
[2,5-NAPS-Trp^9][^{3}H]ACTH	10	12	
[2,5-NAPSS-Trp^9][^{3}H]ACTH	10		
[Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH			21 (10)[b]

[a] For A, BSA (30 μM) and NAPS[^{3}H]ACTH derivative (20 μM) were incubated in 50 mM phosphate buffer (pH 7.4) for 3 h at 22 °C and then photolyzed for 15 min at 0 °C. The BSA–NAPS[^{3}H]-ACTH complex was separated on Sephadex G-75 in 6 M guanidine hydrochloride as shown in Figure 1. B is the same as A except 7.5 μM BSA and 10 μM NAPS[^{3}H]ACTH were used. C is the same as A except 15 μM BSA and 20 μM NAPS[^{3}H]ACTH were used. [b] Same as C except S-carbamidomethylated BSA was used instead of BSA.

reported earlier (Muramoto & Ramachandran, 1980). On the basis of these results, [2,4-NAPS-Trp^9][^{3}H]ACTH was chosen for further studies of the interaction with BSA. At a molar ratio of [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA of 1.33, 28 mol % incorporation was obtained during a 15-min photolysis. The oxidation of the methionine residue in ACTH did not interfere very much with the ability of [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH to form a covalent complex with BSA. However, labeling of BSA decreased considerably upon alkylation of the single cysteine residue in BSA (Table I). The methionine sulfoxide derivative of [2,4-NAPS-Trp^9][^{3}H]-ACTH was employed in experiments involving CNBr cleavage of the complex in order to prevent cleavage of the ACTH molecule. Since ACTH was labeled with tritium on the tyrosine residues 2 and 23, it was desirable to preserve the hormone intact during CNBr cleavage of the complex.

Identification of ACTH Binding Domain of BSA. BSA contains four methionine residues at positions 87, 183, 444, and 546 (Brown, 1975, 1977). The BSA molecule can be split into an amino-terminal fragment containing residues 1–183 and a large carboxy-terminal fragment containing residues 184–582 by CNBr cleavage (King & Spencer, 1970). Only two fragments are formed, even though there are four methionine residues because of the presence of several disulfide bridges in the molecule. Therefore, CNBr cleavage of the covalent complex of ACTH and BSA provides a quick and convenient procedure for ascertaining where the hormone is bound.

[Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH was incubated with BSA at a molar ratio of 1.33 and photolyzed as described in Table I. The covalent complex was isolated by gel filtration as shown in Figure 1 and cleaved with CNBr as described by King & Spencer (1970). The fragments were analyzed by electrophoresis on 15% polyacrylamide slab gels in the presence of $NaDodSO_4$. The results are shown in Figure 2. Peak a represents intact [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex isolated by gel filtration after photolysis. CNBr split this complex into a large fragment (peak b) and a small fragment (peak d) corresponding to the amino-terminal 183 residues. It is apparent that the bulk of the radioactivity is in peaks d and g which correspond to free [^{3}H]ACTH. In the presence of β-mercaptoethanol, the BSA fragments were converted to smaller peptides by reduction of the disulfide bridges. Thus peaks b and d disappear and new peaks, peaks c, e and f, are observed. Peak c is the largest peptide containing 261 amino acid residues derived from the carboxyl CNBr fragment present in peak b. Peaks e and f could not

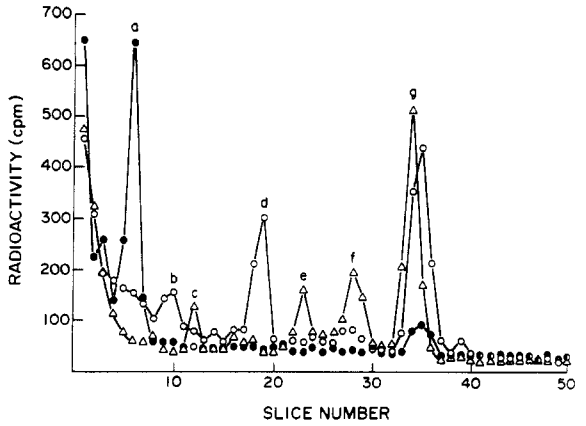

FIGURE 2: $NaDodSO_4$ gel electrophoreses of [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex and its CNBr fragments. [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex (●) and its CNBr fragments were subjected to electrophoresis on 15% polyacrylamide slab gels in $NaDodSO_4$ in the absence (○) and presence (△) of 5% β-mercaptoethanol as described under Materials and Methods.

Table II: Inhibition of Covalent Binding of [2,4-NAPS-Trp^9][^{3}H]ACTH to BSA[a]

inhibitor	% inhibition at 100 μM	500 μM
ACTH	47	78
Ac-NAmPS-Trp	46	58
Ans	81	100
palmitate	−8	−2
indole	23	41
L-Trp	7	21
progesterone	−17	8

[a] BSA (7.5 μM) and [2,4-NAPS-Trp^9][^{3}H]ACTH (10 μM) were incubated in 50 mM phosphate buffer, pH 7.4, in the absence and presence of the inhibitor for 3 h at 22 °C prior to photolysis. The extent of labeling of BSA was measured as described in Figure 1 and Table I.

be assigned since the two peptides have 87 and 96 residues derived from the amino-terminal CNBr fragment and the 102 residues peptide from the carboxy-terminal CNBr fragment comigrated on the gel as shown by Coomassie blue staining.

Peak g observed in the electrophoresis of the CNBr fragments corresponds to free [^{3}H]ACTH. This might have resulted from cleavage of the thioether bond between ACTH and the 2,4-NAPS group or the nitrene-derived linkage between BSA and the hormone during CNBr treatment of the complex. Significant losses of nitrene-derived label upon treatment with CNBr have been reported (Fisher & Press, 1974).

Inhibition of Labeling of BSA by Photoreactive ACTH. These results suggested that [2,4-NAPS-Trp^9][^{3}H]ACTH binds predominantly to the amino-terminal region of BSA. Since the binding sites on BSA for several ligands are known (Brown, 1977), we examined the ability of these ligands to inhibit the photoaffinity labeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH. The results in Table II show that a 10-fold molar excess of ACTH inhibited 47% of the covalent binding of [2,4-NAPS-Trp^9][^{3}H]ACTH to BSA. Ac-NAmPS-Trp, an analogue of NAPS-Trp with an acetylamino group in place of the photoreactive azido group, also caused a 46% inhibition. Among the ligands for which the binding region of BSA is known, indole and Ans were the only ligands which inhibited the covalent attachment of [2,4-NAPS-Trp^9][^{3}H]ACTH to BSA. The binding sites of indole and Ans overlap partially and are located in the amino-terminal domains of BSA (Reed

Table III: Effects of ACTH and Ac-NAmPS-Trp on the Covalent Labeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH[a]

concn of inhibitor[b]	% inhibition of covalent labeling of BSA due to: ACTH	Ac-NAmPS-Trp	ACTH + Ac-NAmPS-Trp
50	38	42	63
100	52	54	73
200	67	63	83
400	73	82	100

[a] BSA (7.5 μM) and [2,4-NAPS-Trp^9][^{3}H]ACTH (10 μM) were incubated in the absence and presence of ACTH or Ac-NAmPS-Trp or both as described in Table II and photolyzed for 15 min at 0 °C. The extent of labeling of BSA was measured as described in Figure 1. [b] For the fourth column, this refers to the concentration of each of the two inhibitors.

Table IV: Inhibition of the Noncovalent Binding of [^{3}H]ACTH to BSA[a]

inhibitor	% inhibition of binding: 100 μM	500 μM
[2,4-NAPS-Trp^9]ACTH	54	70
Ac-NAmPS-Trp	52	65
Ans	58	53
palmitate	6	12
indole	−12	5
L-Trp	−18	−16
progesterone	−40	−38

[a] [^{3}H]ACTH (10 μM) and BSA (7.5 μM) were incubated in 50 mM ammonium acetate buffer, pH 5, in the absence and presence of either 100 or 500 μM inhibitor for 48 h at 4 °C. [^{3}H]ACTH bound to BSA was separated from free [^{3}H]ACTH by gel filtration on a Sephadex G-75 column (0.8 × 29 cm) equilibrated with 100 mM ammonium acetate, pH 5, at 4 °C.

et al., 1975; Brown, 1977). Palmitate and progesterone which are known to bind to the carboxy-terminal domains of BSA did not inhibit the photolabeling of BSA, but slightly enhanced it. These results further support the conclusions derived from analysis of the CNBr fragments and suggest that the photoreactive derivative of ACTH binds to BSA at or near the site where Ans binds.

In order to see if the photoreactive derivatives of ACTH bind to the same region of BSA as ACTH, we investigated the inhibitory actions of ACTH and Ac-NAmPS-Trp alone and in combination on the photolabeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH. This was necessary because Ac-NAmPS-Trp also inhibited the labeling of BSA (Table II). If the ACTH binding site on BSA is different from the binding site for the photoreactive group, an additive effect in the inhibition should be observed in the presence of competitors for both binding sites. The results in Table III show that the covalent labeling of BSA was inhibited by 38% and 42% by ACTH and Ac-NAmPS-Trp, respectively. In the presence of both, the inhibition observed was 63%, considerably less than the additive value of 80%. Similarly, less than additive effects were observed at higher molar excesses of the inhibitors. This indicates partial overlap of the binding sites of ACTH and the NAPS-Trp group.

The above result was confirmed by studies of the noncovalent interaction of [^{3}H]ACTH with BSA. At a 10-fold molar excess, [2,4-NAPS-Trp^9]ACTH, Ac-NAmPS-Trp, and Ans all inhibited the binding of [^{3}H]ACTH to BSA to nearly the same extent (Table IV). Indole and L-Trp which inhibited the photolabeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH did not inhibit the binding of [^{3}H]ACTH. Progesterone caused considerable enhancement of the binding, and palmitate had a very small inhibitory effect.

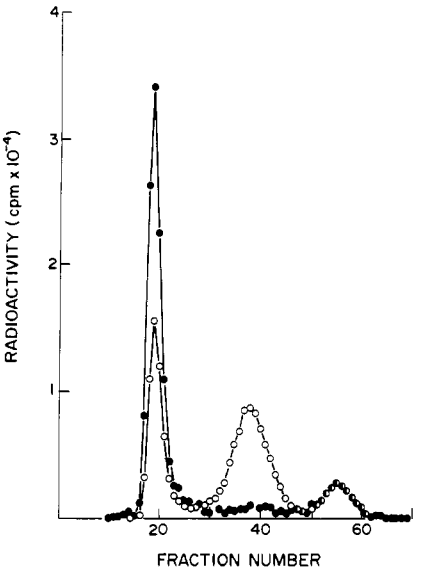

FIGURE 3: Gel filtration of the products of thiolysis of [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex. [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex was chromatographed on Sephadex G-75 equilibrated with 6 M guanidine hydrochloride before (●) and after (○) treatment with 2% β-mercaptoethanol at 37 °C for 48 h. Flow rate, 6 mL/h; fractions, 0.2 mL.

The noncovalent binding of [^{3}H]ACTH to BSA was only 10 mol % in the absence of inhibitors. The photoreactive derivatives were also bound to the same extent as ACTH under these conditions (in the absence of photolysis). However, when [2,4-NAPS-Trp^9][^{3}H]ACTH was photolyzed with BSA under the same conditions (0.1 M ammonium acetate buffer, pH 5), 29 mol % of the derivative was covalently bound. The incorporation of [2,5-NAPS-Trp^9][^{3}H]ACTH into BSA was 11 mol % (data not shown).

Thiolysis and Reduction of Photolyzed NAPS(S)-ACTH–BSA Complexes. It is known that the dinitrophenylsulfenyl (DNPS) group can be removed from DNPS-tryptophan residues by thiolysis (Wilchek & Miron, 1972; Canova-Davis & Ramachandran, 1980). We investigated the removal of ACTH from photolyzed NAPS(S)-ACTH–BSA complexes by thiolysis since this would be of potential value in isolating ACTH receptors. Removal of ACTH from photolabeled receptors would leave the nitrophenyl moiety covalently attached to the receptor.

The [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex obtained by photolysis and gel filtration was treated with β-mercaptoethanol at 37 °C for 48 h. The results in Figure 3 show that the radioactivity associated with BSA decreased and a new peak of radioactivity corresponding to the position of elution of ACTH appeared. The kinetics of thiolysis of photolyzed [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex and photolyzed [2,4-NAPS(S)-Trp^9][^{3}H]ACTH–BSA complex were studied. The mixed disulfide bond of the latter complex was reduced completely within 6 h, but about 20% of [2,4-NAPS-Trp^9][^{3}H]ACTH remained bound to BSA even after 72 h. In the presence of 6 M guanidine hydrochloride, nearly 88% of the specific radioactivity bound to BSA was released from the [2,4-NAPS-Trp^9][^{3}H]ACTH complex.

Discussion

The results show that the photoreactive derivatives of ACTH are covalently attached to BSA by photolysis of mixtures of the two components. Nearly 30 mol % [2,4-NAPS-Trp^9][^{3}H]ACTH was bound to BSA at a molar ratio of 1.33.

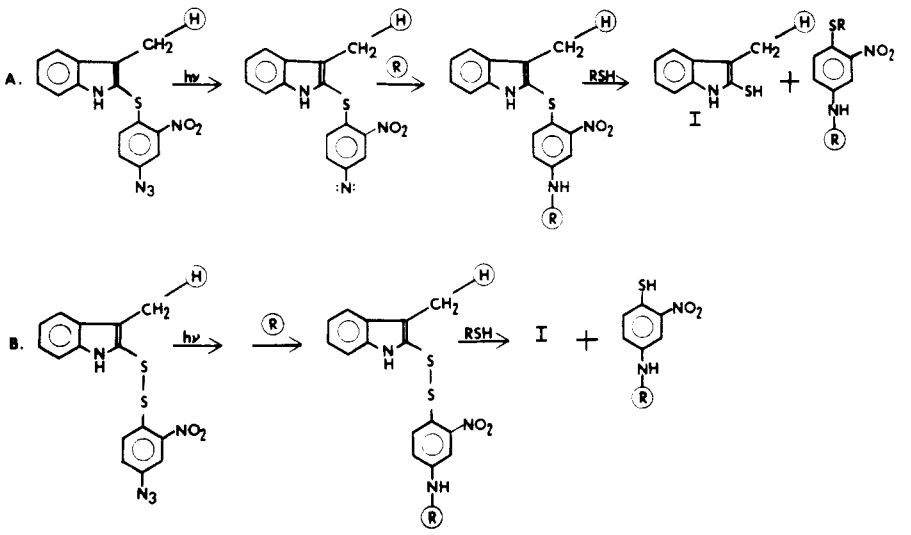

FIGURE 4: Scheme of reactions involved in photoaffinity labeling of BSA and removal of ACTH from the complex. (A) [2,4-NAPS-Trp^9]ACTH. (B) [2,4-NAPSS-Trp^9]ACTH. Circled H, hormone; circled R, BSA or receptor.

On the other hand, only 12 mol % of [2,5-NAPS-Trp^9][^{3}H]ACTH was bound to BSA under identical conditions. This difference may be due to the differences in photoreactivities of the two NAPS-ACTH derivatives. A significant difference between the rates of photolysis of the 2,4-NAPS derivatives and the 2,5-NAPS derivatives was also observed previously when these compounds were photolyzed in the absence of any binding protein (Muramoto & Ramachandran, 1980). The nitrene generated from [2,4-NAPS-Trp^9][^{3}H]ACTH was probably more reactive and, hence, labeled BSA to a greater extent. Owing to the relative low affinity of ACTH for BSA (compared to specific receptors for the hormone), [2,5-NAPS-Trp^9][^{3}H]ACTH may have dissociated from BSA before the lesser reactive nitrene derived from this peptide formed a covalent link with BSA. This may account for the lower degree of labeling with the 2,5-NAPS derivative.

The environment of the photoreactive group also appears to play an important role in determining photoreactivity and, thereby, the efficiency of labeling. We noted previously (Muramoto & Ramachandran, 1980) that the NAPS group in the model compound *N*-Ac-NAPS-Trp-NH_2 was photolyzed more slowly than the NAPS group in ACTH derivatives in aqueous solution. Complete photolysis of [2,5-NAPS-Trp^9]-ACTH in the absence of BSA required more than 60 min whereas maximal labeling of BSA by [2,5-NAPS-Trp^9][^{3}H]ACTH was accomplished in 15 min. These results suggest that the efficiency of photoaffinity labeling of receptors by the NAPS derivatives of ligands is likely to be higher than that observed with model compounds or the ligands alone.

The results in Table I indicate that alkylation of the single cysteine residue in BSA lowers the binding of ACTH derivatives significantly. Carbamidomethylated BSA bound [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH to the extent of 10 mol % whereas intact BSA bound 21 mol % of the same analogue under identical conditions. The latter result suggests that the methionine residue of ACTH may not be an important site for the interaction of the hormone with BSA. Medzhiradszky (1976) found that the methionine sulfoxide analogue of α-melanotropin (α-MSH) bound to BSA with the same affinity as α-MSH. Since α-MSH contains the first 13 residues of ACTH, it is likely that the two peptides bind in a similar manner to BSA.

The ACTH binding domain of BSA was identified by CNBr fragmentation of the covalent complex formed by [Met(O)^4][2,4-NAPS-Trp^9][^{3}H]ACTH and BSA. Most of the radioactivity was associated with the N-terminal 183 residue fragment of BSA, indicating that this portion of the BSA molecule is involved in the interaction with the ACTH derivative. Further localization of the covalent link with BSA was not attempted since proteolytic fragmentation would result in the release of radioactivity associated with the two tyrosine residues at positions 2 and 23 in ACTH. The photoreactive group in ACTH is located between Arg-8 and Lys-15.

Further confirmation of the site of interaction of ACTH with BSA was obtained from studies of the inhibition of the covalent labeling of BSA by various ligands known to bind to specific domains of the protein. These studies showed (Table II) that Ans which binds to the N-terminal domain of BSA was the most effective inhibitor of photolabeling of BSA by [2,4-NAPS-Trp^9][^{3}H]ACTH. Ligands known to bind to the C-terminal domains of BSA, such as palmitate, progesterone, and L-tryptophan, did not inhibit the covalent attachment of the ACTH derivative to BSA. In addition to Ans and indole, both ACTH and Ac-NAmPS-Trp also inhibited the binding of [2,4-NAPS-Trp^9][^{3}H]ACTH to BSA. However, the inhibition due to ACTH and Ac-NAmPS-Trp was not additive (Table III), indicating partial overlap of the binding sites of ACTH and Ac-NAmPS-Trp. The inhibition of the noncovalent interaction of [^{3}H]ACTH to BSA by [2,4-NAPS-Trp^9]ACTH, Ac-NAmPS-Trp, and Ans was found to be comparable (Table IV). All of these results strongly support the findings with CNBr fragments of [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA complex.

On the basis of these studies, it may be inferred that the tryptophan-containing segment of the hormone is probably involved in the interaction with BSA. Since both [^{3}H]ACTH and the radioactive, photoreactive derivatives of ACTH bind noncovalently to BSA to the same extent, it is unlikely that the NAPS group contributes significantly to the interaction. The binding most likely involves hydrophobic interactions between the hormone and the N-terminal domains of BSA.

We have also investigated the removal of ACTH from the covalent complex formed with BSA. This is of considerable interest since removal of the peptide ligand would enable binding studies with the receptor after isolation. Also, removal of the peptide ligand by scission at the thioether or disulfide bridge between the ligand and the photoreactive group would result in the tagging of the receptor protein with the 2-nitro-4(5)-(substituted amino)phenyl group. Since high-affinity

antibodies can be readily generated against such hapten groups, this would provide a simple procedure to isolate the receptor protein by affinity chromatography.

Although both [2,4-NAPS-Trp^9][^{3}H]ACTH–BSA and [2,5-NAPS-Trp^9][^{3}H]ACTH–BSA could be cleaved at the thioether link between the Trp residue of the hormone and the NAPS group (Figure 4) by thiolysis, the reaction was incomplete. Reduction of the disulfide link between the Trp residue of ACTH and the photoreactive group [2,4-NAPSS-Trp^9]-ACTH–BSA proceeded more smoothly, resulting in complete reaction in 6 h. This result eliminates the need for radioactive ligands in photoaffinity labeling. Thus, receptor protein(s) may be covalently labeled with nonradioactive NAPSS derivatives of tryptophan- or cysteine-containing ligands, subjected to reduction with thiols, and then localized on Na-DodSO$_4$ gels or columns by reaction with specific antibodies raised against the haptenic group, 2-nitro-4-(acetylamino)-phenyl. Radioactive protein A may then be used to locate the sites of antibody binding as described by Adair et al. (1978).

Acknowledgments

We thank Professor C. H. Li for his interest.

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Binding of Insulin Receptors to Lectins: Evidence for Common Carbohydrate Determinants on Several Membrane Receptors†

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ABSTRACT: Insulin receptors from human placenta and from cultured lymphocytes (IM-9 line) were solubilized in Triton X-100 and applied to agarose columns containing 12 different immobilized lectins. Receptors from both tissues were adsorbed by lectins that bind *N*-acetylglucosamine (wheat germ), mannose (concanavalin A, lentil, and pea), and galactose (ricins I and II) but were unretained by lectins that bind *N*-acetylgalactosamine (horse gram, *S. Japonica*, and soybean), fucose (gorse seed I), and galactose (*B. simplicifolia* and peanut). After desorption with the appropriate monosaccharides, the insulin-binding capacity of the receptor was increased between 5- and 50-fold with recoveries ranging from 7% to 98%. However, when the solubilized membranes from both tissues were chromatographed sequentially on three different lectin columns (e.g., wheat germ, lentil, and ricin I), the receptor showed only a minor increase in purity after elution from the second and third columns. Receptors for multiplication-stimulating activity (MSA) and epidermal growth factor (EGF) in the solubilized placental membranes behaved very similarly to insulin receptors on sequential lectin chromatography. On the other hand, elution from the lectin columns was followed by a clear increase in the affinity of the receptors as evidenced by (1) a decrease in the concentration of unlabeled insulin, causing half-maximal reduction of [^{125}I]insulin binding, and (2) an increase in bound/free [^{125}I]insulin (tracer binding) greater than the increase in binding capacity (saturation binding). This affinity shift was progressive on sequential lectin chromatography. Our findings indicate that the carbohydrate moiety of the insulin receptor contains *N*-acetylglucosamine, mannose, and galactose but that these saccharide residues are neither receptor nor tissue specific. The increase in affinity of the insulin receptor after its desorption from lectins may be due to separation from the binding site of an associated affinity regulator (inhibitor).

The insulin receptor is an integral membrane protein insofar as detergents are required for its solubilization (Cuatrecasas, 1972). While the binding properties of the membrane-bound and detergent-solubulized receptor are identical and have been defined in considerable detail (Harrison et al., 1978), little is known about its chemical structure. Affinity chromatography on insulin agarose is the logical approach to purification of the insulin receptor. However, this technique gives a poor recovery of functional receptor (Jacobs et al., 1977), in large part due to the fact that insulin affinity columns require harsh denaturing conditions to elute bound receptor.

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