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Photoreactive Derivatives of Corticotropin. 1. Preparation and Characterization of 2,4-Dinitro-5-azidophenylsulfenyl Derivative of Corticotropin†

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ABSTRACT: A photoaffinity probe for corticotropin (ACTH) receptors was prepared by selective modification of the single tryptophan residue in ACTH. A new photoreactive agent, 2,4-dinitro-5-azidophenylsulfenyl chloride, was synthesized and used for introducing the photoreactive group into ACTH. 2,4-Dinitro-5-azidophenylsulfenyl-Trp^9-ACTH (DNAPS-ACTH) was also prepared by thiolysis of 2,4-dinitrophenylsulfenyl-Trp^9-ACTH to form 2-thiol-Trp^9-ACTH and reaction of this with 2,4-dinitro-5-azidofluorobenzene. DNAPS-ACTH was characterized by ultraviolet spectra, peptide mapping, and amino acid analysis. Covalent attachment of DNAPS-ACTH to a pituitary protein fraction FI by photolysis was demonstrated by ultraviolet absorption changes as well as by the use of tritiated DNAPS-ACTH.

Although corticotropin (ACTH)[1] was among the first polypeptide hormones to be utilized in direct binding studies (Lefkowitz et al., 1970), the identification and characterization of specific receptors for ACTH on its target tissues have proved to be a formidable task. In view of the strong tendency of ACTH to bind specifically to a variety of inert materials as well as nonreceptor components of the target tissue, fractionation of the plasma membrane components of the target cell on the basis of binding of the radioactive hormone may result in the isolation of nonreceptor components which are normally present in much higher concentrations than the physiologically relevant receptors. The covalent attachment of the hormone to the receptor under physiological conditions by affinity labeling appears to be a promising approach for the identification of receptors. Conventional chemical affinity labeling, however, is not suitable for this purpose, since the reactive group introduced into the hormone may be destroyed by water or reaction with other nucleophiles on the polypeptide prior to reaching the receptor. Furthermore, this method requires a nucleophilic group at the hormone binding region of the receptor. Both of these limitations can be overcome by the use of a photogenerated species for labeling the receptor (Bayley & Knowles, 1977).

For the identification of the receptor molecule by the method of photoaffinity labeling, it is necessary to perform two modifications on the polypeptide hormone. A suitable radioactive label as well as a photoreactive group must be introduced into the hormone molecule without significantly altering the affinity of the hormone for its physiological receptor. We have already described the successful preparation of specifically tritiated ACTH of high specific radioactivity (90 Ci/mmol) and full biological activity (Ramachandran & Behrens, 1977; Ramachandran et al., 1979). The ϵ-amino group of lysyl residues is the site that is generally modified for the introduction of a photoreactive group (Levy, 1973; Ji, 1977; Das et al., 1977) in peptides. In the case of ACTH, however, both the ϵ-amino group of lysine residues and the D-amino group at the N terminus are unsuitable for modification, since alteration of these sites lowers the biological potency considerably (Ramachandran, 1973). On the other hand, selective chemical modification of the single tryptophan residue in ACTH converts the hormone into a potent antagonist (Ramachandran & Lee, 1970a,b; Moyle et al., 1973). We have investigated the introduction of a photoreactive group at the 2 position of the indole moiety of the tryptophan residue. Previously, we prepared and characterized 2,4-dinitrophenylsulfenyl (DNPS), 2-nitro-4-carboxyphenylsulfenyl (NCPS), and 2-nitrocarbamidophenylsulfenyl (NCMPS)

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[1] Abbreviations used: ACTH, corticotropin; CM-cellulose, carboxymethylcellulose; DNAPS, 2,4-dinitro-5-azidophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; NAPS, 2-nitro-4-azidophenylsulfenyl.

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derivatives of ACTH (Canova-Davis & Ramachandran, 1976). All these derivatives and the *o*-nitrophenylsulfenyl derivative of ACTH (NPS-ACTH; Ramachandran & Lee, 1970a) were found to be potent inhibitors of ACTH-induced adenylate cyclase activation in rat and bovine adrenal plasma membranes (H. Glossmann, C. J. Struck, and J. Ramachandran, unpublished experiments). These results suggested that arylsulfenyl chlorides containing an azido group may prove useful in introducing a photoreactive moiety into the tryptophan of ACTH. The preparation of [2,4-dinitro-5-azidophenylsulfenyl]-Trp^9-ACTH (DNAPS-ACTH) by two different methods, characterization of DNAPS-ACTH, and some of its reactions are described in this paper.

Materials and Methods

Carboxymethylcellulose was purchased from Bio-Rad and Sephadex G-10, G-15, and G-50 were from Pharmacia Fine Chemicals, Inc. Acid protease was purchased from Miles Laboratories, Inc., and leucine aminopeptidase was from Worthington Biochemical Corp.

A Beckman Model DB-GT grating spectrophotometer was used for absorbance measurements at single wavelengths. Continuous spectra were obtained with a Beckman Model DK-2A ratio recording spectrophotometer. Amino acid analyses were performed on a Beckman-Spinco Model 120 amino acid analyzer. Infrared spectra were recorded on a Perkin-Elmer grating spectrophotometer. Radioactivity measurements were performed on a Packard Model 3320 Tri-Carb liquid scintillation spectrometer. Melting points were determined on a Fisher-Johns block and were not corrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

Synthetic human [3,5-^{3}H-Tyr^{2,23}]ACTH was prepared by catalytic dehalogenation of [3,5-diiodo-Tyr^{2,23}]ACTH as described (Ramachandran & Behrens, 1977). The specific radioactivity of this preparation was 90 Ci/mmol.

Porcine ACTH was isolated from clinical grade ACTH generously supplied by Dr. S. H. Eppstein of the Upjohn Company. The product isolated after CM-cellulose chromatography and gel filtration on Sephadex G-50 was found to be homogeneous by paper electrophoresis, amino acid composition, and partition chromatography in 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11 v/v), R_f 0.53. The ability of purified porcine ACTH to stimulate corticosterone synthesis in isolated rat adrenocortical cells was indistinguishable from that of synthetic human ACTH.

Synthesis of 2,4-Dinitro-5-azidophenylsulfenyl Chloride (DNAPS-Cl). Bis(2,4-dinitro-5-aminophenyl) Disulfide (I). Sodium sulfide nonahydrate (12 g, 50 mmol) in 50 mL of 95% ethanol was heated in a round-bottomed flask until the sulfide dissolved. Then 1.6 g of finely divided sulfur was added and heating continued until the sulfur dissolved, forming a brownish red solution of sodium disulfide. Meanwhile, 13.4 g (67 mmol) of 2,4-dinitro-5-fluoroaniline (Aldrich) was suspended in 30 mL of hot 95% ethanol and added to the sodium disulfide solution. The mixture was refluxed for 18 h and cooled to 0 °C. The precipitated product was collected and washed with water (1 L) and 95% ethanol (250 mL). The precipitate was triturated with boiling 95% ethanol and filtered to yield 10 g (70%) of I: mp >300 °C. Anal. Calcd for $C_{12}H_8N_6O_8S_2 \cdot H_2O$ (446.3): C, 32.27; H, 2.26; N, 18.83. Found: C, 32.48; H, 2.16; N, 18.72.

Bis(2,4-dinitro-5-azidophenyl) Disulfide (II). The disulfide I (2.23 g, 5 mmol) was triturated into solution with 10 mL of concentrated H_2SO_4 and stirred at −10 to 0 °C. Sodium nitrite (0.9 g, 13 mmol) in water (5 mL) was added dropwise over 15 min, and stirring was continued for 45 min. Ice-cold water (50 mL) was added, followed by sodium azide (1.1 g, 17 mmol) in water (5 mL) dropwise. After all the azide was added, the mixture was stirred for another 15 min at −10 to 0 °C. The product was filtered, washed with ice-cold water, and air-dried. The product was then triturated with $CHCl_3$ and filtered from significant amounts of unreacted I. The orange-red solution was kept at −20 °C for 48 h in the dark. The crystalline product was filtered and washed with $CHCl_3$–petroleum ether (1:4 v/v). II was obtained in 20% yield: mp 90 °C dec; IR (Nujol) 2150 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{12}H_4N_{10}O_8S_2$ (480.97): C, 29.94; H, 0.85; N, 29.13. Found: C, 30.33; H, 1.09; N, 25.7.

The low nitrogen value indicated that III may be decomposing. A high-resolution mass spectrum of freshly prepared II gave a peak mass of 480.9744 ± 10 ppm. The expected *m/e* value of 480.9733 is in good agreement.

2,4-Dinitro-5-azidophenylsulfenyl Chloride (DNAPS-Cl). The disulfide VI (0.2 g) was suspended in 15 mL of CCl_4 and saturated with chlorine gas dried through a sulfuric acid drying tower. The reaction mixture was warmed to 45 °C, 0.1 mL of 20% fuming sulfuric acid was added, and the mixture was stirred for 2 h. Some tar from the charring action of the sulfuric acid was evident. The warm solution was filtered, cooled, and evaporated (Kharasch et al., 1950). The residual dark red oil was triturated with ether and desiccated over P_2O_5 in the dark. The product migrated as a single yellow spot in thin-layer chromatography in both $CHCl_3$–CH_3COOH (15:1 v/v), R_f 0.89, and $CHCl_3$–CH_3COCH_3 (1:1 v/v), R_f 0.77. DNAPS-Cl was obtained in low yield (~15%): mp 87 °C dec; IR (CCl_4) 2110 cm^{-1}, N_3 asymmetric stretch. The DNAPS-Cl was found to decompose on storage. It was prepared fresh and used immediately for the modification of peptides.

Preparation of the DNAPS Derivative of N-Acetyltryptophanamide. To a solution of 50 mg of *N*-acetyltryptophanamide in 2 mL of glacial acetic acid, 50 mg of DNAPS-Cl was added at room temperature and allowed to react in the dark overnight. The product was precipitated by the addition of 30 mL of water, collected, and dried in vacuo over P_2O_5. Since difficulty was encountered in obtaining a crystalline compound, the product was eluted with $CHCl_3$ from thin-layer chromatography in 1-butanol–HOAc–H_2O (4:1:1 v/v), R_f 0.86. The material recovered in this manner decomposed at 140 °C. Thin-layer chromatography revealed a single yellow spot in chloroform–acetic acid (15:1 v/v), R_f 0.45.

Preparation of DNAPS-ACTH. Fifteen milligrams of ACTH was dissolved in 0.2 mL of water containing a 100-fold molar excess of methionine, and 1.8 mL of glacial acetic acid was added. Twenty milligrams of the DNAPS-Cl in 1.0 mL of glacial acetic acid was added, and the solution was kept at room temperature in the dark with occasional shaking. After 4 h the solution was diluted with 30 mL of water and extracted with ethyl acetate. After the organic phase was extracted with 0.1% acetic acid, the combined aqueous phase was lyophilized. The peptide was then separated from any further excess reagent by exclusion chromatography on Sephadex G-10 (1.5 × 19 cm) in 0.1 N acetic acid. The derivative was purified by chromatography on a CM-cellulose column (1 × 25 cm) using an ammonium acetate gradient as described for ACTH (Pickering et al., 1963).

Tritiated ACTH (28 μg) was reacted with a 20-fold molar excess of DNAPS-Cl in 90% acetic acid in the presence of a 100-fold molar excess of methionine for 4 h with occasional

shaking in the dark. The incubation mixture was chromatographed on a Sephadex G-15 column (0.7 × 18.4 cm) equilibrated with 90% acetic acid. The modified hormone was monitored by its radioactivity, and the appropriate fractions were pooled and lyophilized. The hormone derivative was stored in 0.1 N acetic acid at −20 °C: yield, 70%.

Conversion of DNPS-ACTH to DNAPS-ACTH. Two milligrams of DNPS-ACTH (Canova-Davis Ramachandran, 1976) was dissolved in 0.2 N acetic acid, and the pH was adjusted to 8.0 with NH_3. Nitrogen was bubbled through the mixture for 30 min, and a 100-fold molar excess of β-mercaptoethanol was added. The mixture was capped and incubated at room temperature in the dark for 2 days. The reaction mixture was then extracted with ethyl acetate (1 mL × 3) and adjusted to pH 5.0. A 10-fold molar excess of aryl azide was added and incubated at room temperature. The reaction was terminated by extraction of the excess reagent with ether or ethyl acetate and adjustment of the pH to approximately 2 with HCl. The peptide was purified on a Sephadex G-25 column equilibrated with 0.1 N acetic acid.

Photolysis. Irradiation was conducted in a Rayonet photochemical reactor using the RUL 3000-Å ultraviolet lamps. Solutions were placed in Pyrex containers and kept at 0 °C by means of an ice bath. The spectral properties of the Pyrex tubes were as follows: less than 1% transmission at 254 nm where the lamps have a relatively high emission; 55% transmission at the predominant emission wavelength of 300 nm.

Photolysis of DNAPS-ACTH–Binding Protein Complexes. Binding protein–DNAPS-ACTH complexes were prepared by equilibration in a 4.5:1 ratio by weight in 0.1 M NH_4OAc, pH 4.3 or 9.4, and photolyzed at 0 °C for 2 h. The complexes were then chromatographed on a Sephadex G-50 column (1.5 × 22.4 cm) equilibrated with 1 M formic acid to facilitate dissociation of noncovalently bound peptides.

Enzymatic Digestion. The peptide (0.5 mg) was incubated with 50 μg of acid protease in 0.2 mL of 0.01 N HCl at 37 °C for 20 h. After lyophilization, the sample was incubated with 1.6 U of leucine aminopeptidase in 0.2 mL of 0.05 M Tris-HCl buffer (pH 8.5), containing 0.01 M $MgCl_2$, for 24 h at 37 °C. The sample was then analyzed on the amino acid analyzer.

Peptide Maps. The peptide (1 mg) was incubated with diphenylcarbamoyl chloride treated trypsin (20 μg) in 0.2 mL of 0.2 M ammonium acetate (pH 8.0) at 37 °C for 8 h. After lyophilization, the digest was subjected to descending chromatography on Whatman No. 3MM paper in the upper phase of freshly prepared 1-butanol–acetic acid–water (4:1:5 v/v) for 20 h at room temperature. High-voltage electrophoresis was performed in the second dimension at pH 2.1 in the buffer composed of 90% formic acid–acetic acid–water (218:63:719 v/v) for 50 min (35 V/cm). The peptide map was examined visually for yellow spots and then developed with ninhydrin.

Results

Synthesis of DNAPS-Cl. The synthetic scheme employed for the preparation of other nitrophenylsulfenyl chlorides (Hubacher, 1935; Havlik & Kharasch, 1955) was adapted for the synthesis of DNAPS-Cl. Although disulfide I was obtained in good yield, because of the insolubility of I, the azido disulfide II was obtained in low yield. Both II and DNAPS-Cl, which contain the photosensitive azido group, were found to be marginally stable at room temperature. In view of this, DNAPS-Cl was prepared fresh and used immediately.

Characterization of DNAPS-ACTH Prepared by Direct Sulfenylation. Introduction of the photoreactive DNAPS group into ACTH was accomplished by allowing the hormone to react with DNAPS-Cl in 90% acetic acid. An excess of methionine was included in the reaction mixture to act as a scavenger and protect the methionine residue in the hormone from oxidation (Canova-Davis & Ramachandran, 1976). No evidence of unreacted ACTH was seen when DNAPS-ACTH was subjected to ion exchange chromatography on carboxymethylcellulose.

Table I: Amino Acid Composition of ACTH and DNAPS-ACTH[a]

amino acid	ACTH	DNAPS-ACTH	amino acid	ACTH	DNAPS-ACTH
Trp	1.21		Gly	3.19	1.25
Lys	3.61	2.00	Ala	3.33	3.81
His	1.00	0.88	Val	2.95	2.55
Arg	3.00	3.00	Met	1.02	0.78
Asp	1.55	1.28	Met SO		
Ser + Asn	2.38	2.77	Leu	2.15	2.59
Glu	5.00	5.00	Tyr	2.12	1.89
Pro	4.06	2.26	Phe	3.12	3.19

[a] The peptides were digested with acid protease followed by leucine aminopeptidase as described under Materials and Methods.

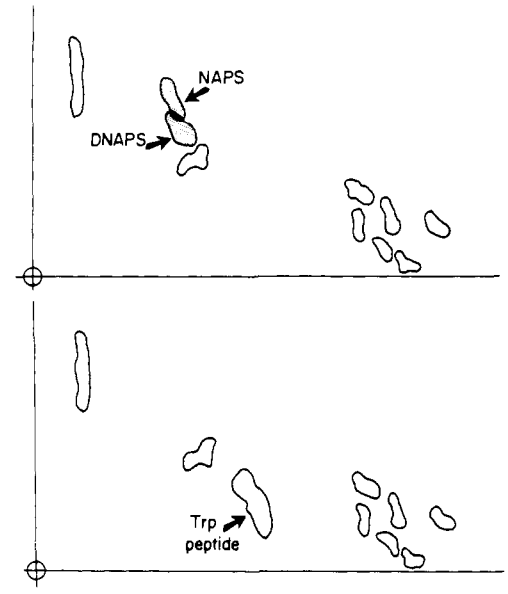

FIGURE 1: Peptide maps of ACTH and photoreactive ACTH derivatives. The peptides were digested with trypsin, and the fragments were separated on paper as described under Materials and Methods. The lower panel is the fingerprint of ACTH. The new positions of the tryptophan peptide in the maps of the modified ACTH derivatives are indicated in the upper panel. The preparation and characterization of 2-nitro-4-azidophenylsulfenyl-Trp[9]-ACTH (NAPS-ACTH) are described in the accompanying article (Muramoto & Ramachandran, 1980).

The purified DNAPS-ACTH was subjected to enzymatic digestion with acid protease followed by leucine aminopeptidase in order to assess the completeness and selectivity of the reaction. As seen from the results in Table I, the tryptophan residue in DNAPS-ACTH appears to be completely modified. Methionine was found to be intact, and no methionine sulfoxide could be detected. The low values for lysine, proline, and glycine in the digest of DNAPS-ACTH compared to ACTH suggest that modification of the Trp residue renders digestion of the segment Trp-Gly-Lys-Pro difficult. Peptide maps of tryptic digests of DNAPS-ACTH and ACTH were identical except for the position of the tryptophan-containing peptide (Figure 1). The Trp peptide was missing in the map of the modified hormone. Instead, a single, new, yellow ninhydrin positive spot was observed. The peptides corresponding to the N-terminal octapeptide and the modified Trp peptide were eluted with 0.1 N ammonia, hy-

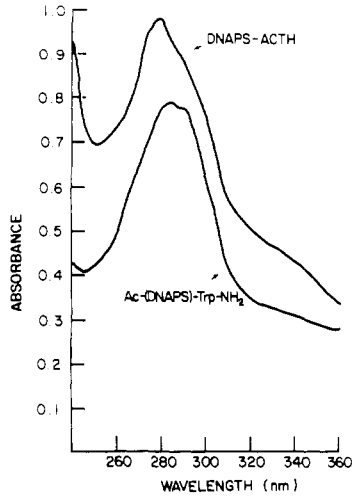

FIGURE 2: Ultraviolet absorption spectra of Ac-(DNAPS)-Trp-NH_2. The spectrum of DNAPS-ACTH was recorded in 0.1 N acetic acid; that of the Ac-(DNAPS)-Trp-NH_2 was recorded in 95% ethanol.

Table II: Molar Absorptivities of DNAPS-Modified Compounds

compound	λ (nm)	E
Ac-(DNAPS)-Trp-NH_2[a]	282	17 700
	360	6 300
DNAPS-ACTH[b]	278	15 000
	360	5 900

[a] The spectrum was recorded in 95% ethanol. [b] The spectrum was recorded in 0.1 N HOAc.

drolyzed with acid, and subjected to amino acid analysis. The Pauly positive N-terminal peptide had the following amino acid composition: $His_{0.88}Arg_{0.91}Ser_{1.64}Gln_{1.00}Met_{0.97}Tyr_{0.79}Phe_{0.89}$. That of the yellow Trp peptide was: $Lys_{1.74}Pro_{1.04}Gly_{2.25}Val_{1.00}$. These results strongly suggest that DNAPS-ACTH was selectively modified at a single site, namely tryptophan.

Further evidence of modification at a single site in the hormone was obtained from ultraviolet absorption spectra (Figure 2). The molar absorptivities of DNAPS-ACTH and the model compound acetyl-2,4-dinitro-5-azidophenylsulfenyltryptophanamide are given in Table II. Both compounds exhibited a maximum around 280 nm. There was no maximum at 360 nm, but the extinction coefficients at this wavelength are an index of the number of DNAPS groups. It is apparent that the model compound containing one DNAPS group and DNAPS-ACTH have comparable molar absorptivities at 360 nm, confirming substitution at a single site in ACTH.

Preparation of DNAPS-ACTH by Modification of 2-Thiol-Trp^9-ACTH. An alternative approach to the synthesis of DNAPS-ACTH involves introduction of a thiol group at the 2 position of the indole moiety of Trp-9 in ACTH and reaction of the thiol-Trp^9-ACTH with 2,4-dinitro-5-azidofluorobenzene. The quantitative removal of 2,4-dinitrophenyl groups from 2,4-dinitrophenylsulfenyltryptophan residues in model compounds and proteins by thiolysis was reported by Wilchek & Miron (1972). Treatment of DNPS-Trp compounds with β-mercaptoethanol results in the formation of 2-thioltryptophan derivatives.

DNPS-ACTH prepared according to Canova-Davis & Ramachandran (1976) was incubated with a 100-fold molar excess of β-mercaptoethanol at pH 8.0 at room temperature, and thiolysis was followed spectrophotometrically. As reported by Wilchek & Miron (1972), an increase in the absorption was seen at 313 nm. The reaction was complete after 44 h. After extraction of the byproduct *S*-dinitrophenyl-2-

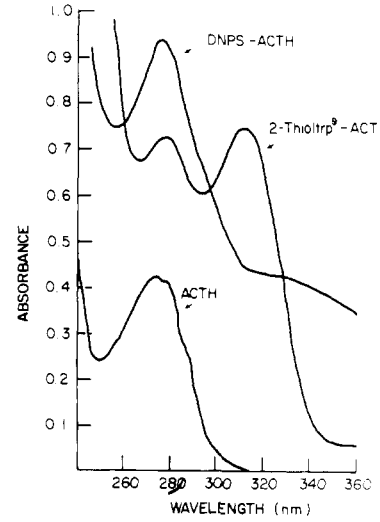

FIGURE 3: Ultraviolet spectra of ACTH, DNPS-ACTH, and 2-thiol-Trp^9-ACTH. The spectra of ACTH and DNPS-ACTH were recorded in 0.001 N HCl, and that of 2-thiol-Trp^9-ACTH was recorded 0.1 N NH_4HCO_3, pH 8.0.

mercaptoethanol with ethyl acetate, the ultraviolet absorption spectrum of 2-thiol-Trp^9-ACTH was determined. As seen in Figure 3, the spectrum of 2-thiol-Trp^9-ACTH is different from both DNPS-ACTH and the unmodified hormone, ACTH.

Chemical proof of the formation of 2-thiol-Trp^9-ACTH was obtained by reacting the peptide with a 10-fold molar excess of 1-fluoro-2,4-dinitrobenzene at pH 5.0 for 30 min. DNPS-ACTH was regenerated as judged by the ultraviolet spectrum and peptide map (data not shown). Furthermore, reaction of 2-thiol-Trp^9-ACTH with 5,5′-dithiobis(2-nitrobenzoic acid) (NbS_2, Ellman's reagent) also confirmed the presence of a sulfhydryl group (Ellman, 1959).

Reaction of 4-fluoro-3-nitrophenylazide with 2-thiol-Trp^9-ACTH failed to modify the peptide. Increased concentrations of reagent, longer reaction times, and high pH were ineffectual. This result is consistent with the report of Fleet et al. (1972) that 4-fluoro-3-nitrophenylazide reacts with methylamine approximately 1000-fold less rapidly than with 2,4-dinitrofluorobenzene. The reagent 2,4-dinitro-5-azidofluorobenzene, on the other hand, is reported to react with amino groups twice as rapidly as 2,4-dinitrofluorobenzene (Wilson et al., 1975). We synthesized 2,4-dinitro-5-azidofluorobenzene from 2,4-dinitro-5-aminofluorobenzene as described (Wilson et al., 1975) and reacted it with 2-thiol-Trp^9-ACTH at pH 5.0. Formation of DNAPS-ACTH was complete in 15 min with a 10-fold molar excess of the reagent as judged by spectral and electrophoretic analysis. The ultraviolet spectrum of the product after removal of excess reagent by extraction with ethyl acetate was the same as that of DNAPS-ACTH prepared by direct modification of ACTH with DNAPS-Cl (Figure 2). Electrophoreis at pH 3.7 on Whatman No. 3MM paper for 3 h (12 V/cm) revealed a single yellow ninhydrin and Pauly positive peptide migrating in the same position as DNAPS-ACTH.

Photolysis of DNAPS-ACTH Complex with a Pituitary Binding Protein. Irradiation of DNAPS-ACTH at 0 °C in a Rayonet photochemical reactor caused spectral changes centered around 290 nm as well as 360 nm. In order to check whether covalent attachment of the hormone can be obtained by photolysis, DNAPS-ACTH was incubated with a crude ACTH binding protein fraction FI obtained from ovine pituitary glands as described (Canova-Davis & Ramachandran, 1976). Photolysis of this mixture resulted in covalent attachment of DNAPS-ACTH to FI. The DNAPS-ACTH–FI

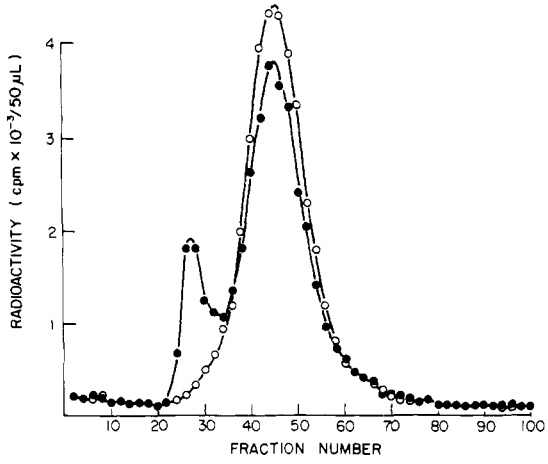

FIGURE 4: Gel filtration of the tritiated DNAPS-ACTH–FI complex. One milligram of FI protein was mixed with 30 nmol of tritiated DNAPS-ACTH (sp act. 50 000 cpm/nmol) in 1 mL of 1 M formic acid. One-half of the mixture was chromatographed directly on a Sephadex G-50 column (1.5 × 22.4 cm) equilibrated with 1 M formic acid (○). The pH of the remaining mixture of FI and [^{3}H]-DNAPS-ACTH was adjusted to 5.0, and the mixture was incubated at 0 °C for 16 h in the dark and photolyzed for 1 h at 0 °C. The photolyzed reaction mixture was then chromatographed on the Sephadex G-50 column (●): flow rate, 12 mL/h; fractions, 0.75 mL/tube.

complex was chromatographed on Sephadex G-50 equilibrated with 1 M formic acid to dissociate noncovalently bound DNAPS-ACTH. The ratio of absorbance at 360 and 280 nm of FI (which is eluted at the void volume) increased from 0.064 before photolysis to 0.168 after photolysis, suggesting that the ACTH derivative that absorbs at 360 nm migrated with FI upon gel filtration. This was also demonstrated by photolyzing tritium-labeled DNAPS-ACTH with FI (Figure 4). It is apparent that radioactivity is associated with FI after photolysis even under strongly dissociating conditions. From the results shown in Figure 4, it can be estimated that approximately 20% of the tritiated DNAPS-ACTH became attached covalently to FI upon photolysis. This amounts to 4 nmol of [^{3}H]DNAPS-ACTH bound per 0.5 mg of FI. The covalent complex of FI with [^{3}H]DNAPS-ACTH was pooled, lyophilized, and treated with β-mercaptoethanol at pH 8.0 for 48 h at room temperature. When this mixture was lyophilized and rechromatographed on Sephadex G-50, the radioactivity was no longer associated with FI but emerged in the position of ACTH (data not shown). Thus, the radioactive hormone could be released from the covalent FI complex by thiolysis.

Discussion

Previous studies on the structure–function relationships of ACTH have shown that the single tryptophan residue in the hormone is located at a crucial site that is most likely to be intimately involved in the interaction of the hormone with the receptor. [For a review, see Ramachandran (1973).] Furthermore, introduction of different substituted aryl sulfenyl groups into the 2 position of the indole group of the tryptophan appears to convert the hormone into potent antagonists (Canova-Davis & Ramachandran, 1976; H. Glossmann, C. J. Struck, and J. Ramachandran, in preparation). Therefore, we have prepared a photosensitive derivative of ACTH by introducing the 2,4-dinitro-5-azidophenylsulfenyl group into the 2 position of the indole of Trp-9 in ACTH. This was accomplished in two ways. The first method was the direct reaction of ACTH with 2,4-dinitro-5-azidophenylsulfenyl chloride under acidic conditions. DNAPS-Cl was synthesized by a scheme analogous to that used for the preparation of other substituted aryl sulfenyl chlorides (Havlik & Kharasch, 1955). DNAPS-ACTH was purified by gel filtration and ion exchange chromatography. That selective modification at the 2 position of indole in Trp-9 was achieved was established by amino acid analysis of an enzyme digest of DNAPS-ACTH, peptide mapping of a tryptic digest, and ultraviolet absorption spectra of the modified hormone.

DNAPS-ACTH was also prepared by reaction of 2,4-dinitro-5-azidofluorobenzene with 2-thiol-Trp^9-ACTH. The latter was prepared by thiolysis of DNPS-ACTH with β-mercaptoethanol. The removal of the dinitrophenyl group from DNPS-ACTH and the formation of 2-thiol-Trp^9-ACTH were established by spectral analysis, sulfhydryl group assay by Ellman's reagent, and by regeneration of DNPS-ACTH by reaction with 2,4-dinitrofluorobenzene. DNAPS-ACTH prepared from 2-thiol-Trp^9-ACTH had the same spectral and electrophoretic properties as DNAPS-ACTH prepared by direct reaction with DNAPS-Cl.

The presence of a photosensitive group in DNAPS-ACTH was revealed by the alteration in the ultraviolet absorption spectrum after photolysis. The ability of DNAPS-ACTH to form covalent bonds with interacting proteins was demonstrated by photolyzing a complex of DNAPS-ACTH with a crude ovine pituitary ACTH binding protein, FI. Spectral analysis of FI after photolysis and gel filtration under dissociating conditions revealed that the photoreactive ACTH had been attached covalently to FI. This was also observed when tritiated DNAPS-ACTH was complexed with FI and photolyzed. These results clearly show that DNAPS-ACTH can be covalently attached to proteins to which it binds.

DNAPS-ACTH has already proved to be a valuable tool in identifying specific receptors for ACTH. We have utilized tritiated DNAPS-ACTH for labeling ACTH receptors on rat adipocyte membranes (Ramachandran et al., 1979, 1980). DNAPS-ACTH was found to be a competitive inhibitor of ACTH-induced adenylate cyclase in rat adipocyte ghosts. When tritiated DNAPS-ACTH was incubated with rat adipocyte plasma membranes and photolyzed for 10 min at 0 °C, 20% of the specific binding sites were covalently labeled as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No incorporation of radioactivity was observed when the photolysis was conducted in the presence of excess ACTH. Thus, it is clear that DNAPS-ACTH is a useful photoaffinity probe for ACTH receptors.

The instabilities of the DNAPS-Cl as well as the DNAPS peptides, however, limit the utility of this photosensitive group. It is necessary to prepare the reagent fresh, use it for modification immediately, and conduct the photolysis without delay. Furthermore, the presence of a nitro group in the ortho position to the azido group may contribute to a significant degree of benzofuroxan formation (Bailey & Case, 1958) and thus decrease the efficiency of photolabeling by the nitrene. A more stable photoreactive probe may be obtained by the use of 2-nitro-4-azidophenylsulfenyl chloride or 2-nitro-5-azidophenylsulfenyl chloride. The synthesis of these reagents and their use in modification of ACTH are described in the accompanying paper (Muramoto & Ramachandran, 1980).

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Photoreactive Derivatives of Corticotropin. 2. Preparation and Characterization of 2-Nitro-4(5)-azidophenylsulfenyl Derivatives of Corticotropin†

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ABSTRACT: Two new photoreactive arylsulfenyl chlorides, 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl), have been synthesized and used for the selective modification of corticotropin (ACTH). Both reagents reacted rapidly with *N*-acetyltryptophanamide and ACTH under acidic conditions. The NAPS derivatives of ACTH were purified by partition chromatography and characterized by absorption spectra, amino acid analysis, and peptide mapping. The spectral changes caused by photolysis as well as the kinetics of photolysis are described. Tritiated 2,5-NAPS-ACTH was attached covalently to a pituitary protein fraction FI by photolysis. The photolabeling of FI was blocked in the presence of excess ACTH.

In order to prepare photoreactive derivatives of the pituitary hormone corticotropin (ACTH),[1] we have investigated the synthesis and use of arylsulfenyl chlorides incorporating an azido group. Arylsulfenyl chlorides have been found to be specific mild reagents for the modification of tryptophan and cysteine residues of polypeptides in acidic media (Scoffone et al., 1968; Fontana et al., 1968; Canova-Davis & Ramachandran, 1976). Tryptophan is converted into a derivative with thioether function in the 2 position of the indole nucleus, and cysteine is converted to an unsymmetrical disulfide. In aqueous alkaline solution, sulfenyl chlorides react with α-amino groups of amino acids and peptides as well as ϵ-amino groups of lysine residues (Zervas et al., 1963). Hence, a variety of photoreactive derivatives of polypeptides and proteins may be obtained by selective modification with arylsulfenyl chlorides containing azido groups. Our initial studies revealed that the preparation of 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) was more difficult than the synthesis of 2,4-dinitro-5-azidophenylsulfenyl chloride (DNAPS-Cl). Therefore, we synthesized DNAPS-Cl and used it for the modification of ACTH. The preparation and characterization of DNAPS-Trp^9-ACTH and its use in photoaffinity labeling have been described in the previous article (Canova-Davis & Ramachandran, 1980) and elsewhere (Ramachandran et al., 1979, 1980). However, these studies showed that the DNAPS group is unstable. The presence of a nitro group adjacent to the azido group may also decrease the efficiency of photoaffinity labeling because of the formation of benzofuroxan derivatives (Bailey & Case, 1958). It became apparent that a more stable photoreactive arylsulfenyl chloride would be highly useful in preparing photoaffinity labels for ACTH receptors in particular and for the introduction of photoreactive groups into proteins in general. We have developed procedures for

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[1] Abbreviations used: ACTH, corticotropin; NAPS, 2-nitro-4-azidophenylsulfenyl; DNAPS, 2,4-dinitro-5-azidophenylsulfenyl.

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