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Preparation of Photoreactive Derivatives of Glutathione and [9-(2-Mercaptotryptophan)]corticotropin by Selective Modification of the Sulfhydryl Group†

Koji Muramoto and J. Ramachandran*

ABSTRACT: The photoreactive arylsulfenyl chlorides 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl) have been used for the selective modification of thiol groups in glutathione and [Trp(SH)^9]corticotropin (ACTH). Both reagents reacted rapidly with both types of thiol groups to form unsymmetrical disulfides. The photoreactive derivatives of glutathione and [Trp(SH)^9]ACTH were stable to neutral and acidic conditions but were readily cleaved above pH 9 and by β-mercaptoethanol. Photolysis of the NAPS derivatives of [Trp(SH)^9]-ACTH at neutral pH resulted in the formation of covalently linked polymers and dimers which yielded monomer upon treatment with β-mercaptoethanol. Analysis of the amino acid composition of acid hydrolysates of photolyzed monomeric and dimeric products indicated a decrease in proline, valine, tyrosine, and phenylalanine.

Recently, we described the synthesis of two new photoreactive arylsulfenyl chlorides and their use for selective modification of tryptophan residues in peptides (Muramoto & Ramachandran, 1980). The reagents 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl)[1] and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl) were used to introduce a photoreactive group into the tryptophan residue of the pituitary hormone corticotropin (ACTH). The modified hormone 2,5-NAPS-Trp^9-ACTH was successfully employed for photoaffinity labeling of ACTH receptors on isolated rat adrenocortical cells (Ramachandran et al., 1980). Since arylsulfenyl chlorides are known to react with sulfhydryl groups also (Fontana et al., 1968), we have investigated the utility of 2,4-NAPS-Cl and 2,5-NAPS-Cl for the selective modification of sulfhydryl groups in peptides. In this article, we describe the selective modification of thiol groups of reduced glutathione and [Trp(SH)^9]ACTH as well as the photoreactivities of the modified peptides.

Materials and Methods

Highly purified porcine ACTH was prepared as previously described (Canova-Davis & Ramachandran, 1976). The syntheses of 2,4-NAPS-Cl and 2,5-NAPS-Cl have been reported previously (Muramoto & Ramachandran, 1980).

Preparation of the 2,4- and 2,5-NAPS Derivatives of Reduced Glutathione. To a solution of 120 mg (0.40 mmol) of reduced glutathione (Calbiochem) in 6 mL of glacial acetic acid was added 100 mg (0.44 mmol) of NAPS-Cl. The reaction mixture was stirred for 2 h at room temperature in the dark and poured into 100 mL of ethyl ether. The precipitate was filtered and washed with ethyl ether. NAPS-glutathione suspended in 50 mL of water was collected, washed with water and ethyl ether, and dried in vacuo: 2,4-NAPS-glutathione, yield, 120 mg (61%); mp 195 °C dec; TLC on silica gel G, R_f 0.36 in 1-butanol–acetic acid–water (4:1:1 v/v) (solvent A); IR (Nujol) 2090–2060 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{16}H_{19}N_7O_8S_2$ (M_r 501.5): C, 38.32; H, 3.82; N, 19.55. Found: C, 38.25; H, 4.01; N, 19.72. 2,5-NAPS-glutathione, yield, 180 mg (92%); mp 185 °C dec; TLC on silica gel G, R_f 0.36 in solvent A; IR (Nujol) 2100 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{16}H_{19}N_7O_8S_2$: C, 38.32; H, 3.82; N, 19.55. Found: C, 37.90; H, 3.99; N, 19.35.

Kinetics of Reaction of NAPS-chlorides with Reduced Glutathione. Equal volumes (2.5 mL) of a 20 mM solution of reduced glutathione in 90% acetic acid and 40 mM 2,4- or 2,5-NAPS-Cl in glacial acetic acid were mixed at 21 °C. At various times, 0.1 mL of the reaction mixture was added to 3 mL of water. Excess reagent was extracted with 3 mL of ethyl acetate. The aqueous phase (0.5 mL) was diluted with 1 mL of glacial acetic acid. The increase in absorbance at

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[1] Abbreviations used: ACTH, corticotropin; 2,4(5)-NAPS-Cl, 2-nitro-4(5)-azidophenylsulfenyl chloride; 2,4-DNPS-ACTH, [2,4-dinitrophenylsulfenyl-Trp^9]ACTH; [2,4(5)-NAPSS-Trp^9]ACTH, 2-nitro-4(5)-azidophenylsulfenyl derivatives of [Trp(SH)^9]ACTH; [Trp(SH)^9]corticotropin, [9-(2-mercapto)tryptophan)]corticotropin.

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380 nm for 2,4-NAPS-glutathione and at 320 nm for 2,5-NAPS-glutathione was measured. Control solutions containing no glutathione were prepared in the same way for reference.

Stability of the Disulfide Bond of NAPS-glutathione. About 3 mg of NAPS-glutathione was dissolved in 50 mL of distilled water. Equal volumes (1.5 mL) of a sample solution and buffer solution were mixed quickly in a cuvette. The decreases in absorbance at 380 nm for 2,4-NAPS-glutathione and at 320 nm for 2,5-NAPS-glutathione were measured at 21 °C.

Preparation of NAPS Derivatives of [Trp(SH)^9]ACTH. [2,4-Dinitrophenylsulfenyl-Trp^9]ACTH was prepared according to Canova-Davis & Ramachandran (1980). [2,4-DNPS-Trp^9]ACTH (20 mg) was taken in a centrifuge tube and dissolved in 1 mL of 0.01 M acetic acid. β-Mercaptoethanol (0.2 mL) and 19 mL of 0.1 M ammonium bicarbonate buffer (pH 8.5) were successively added. The mixture was stirred gently for 24 h at room temperature under a nitrogen atmosphere. The progress of thiolysis was followed by spectral changes and by TLC on silica gel G. [Trp(SH)^9]ACTH showed R_f 0.41 in 1-butanol–pyridine–acetic acid–water (5:5:1:4 v/v) (solvent B) and [2,4-DNPS-Trp^9]ACTH, R_f 0.45 (solvent B). The reaction mixture was acidified with 5 mL of 2 N acetic acid and extracted with 20 mL of ethyl acetate twice to remove *S*-(2,4-dinitrophenyl)-2-mercaptoethanol. The lyophilized [Trp(SH)^9]ACTH and L-methionine (200 mg) were dissolved in 6 mL of 90% acetic acid and divided into two portions. 2,4- or 2,5-NAPS-Cl (35 mg) was added to each solution, and the reaction was allowed to proceed for 2 h at room temperature in the dark. The reaction mixtures were extracted with ethyl acetate after dilution with 40 mL of distilled water. After lyophilization, the 2,4- and 2,5-NAPS derivatives of [Trp(SH)^9]ACTH were purified by gel filtration on Sephadex G-25 (1.5 × 60 cm) equilibrated with 0.1 M formic acid. 2,4- and 2,5-NAPS derivatives of [Trp(SH)^9]-ACTH were obtained in 75% (7.9 mg) and 85% (9.0 mg) yield, respectively. They were homogeneous as shown by TLC on silica gel G in solvent B, R_f 0.43.

Photolysis of NAPS Derivatives of [Trp(SH)^9]ACTH ([NAPSS-Trp^9]ACTH). [NAPSS-Trp^9]ACTH (1.5 mg) dissolved in 0.1 mL of 0.1 M acetic acid was diluted with 2.5 mL of 0.1 M phosphate buffer (pH 7.4). Photolyses were performed for 15 min at 0 °C as described previously (Muramoto & Ramachandran, 1980). The photolyzed [NAPSS-Trp^9]ACTH was lyophilized. Half of the photolyzed product was dissolved in 0.5 mL of 1.6 M formic acid and subjected to gel filtration on a Sephadex G-50 (1 × 46 cm) column equilibrated with 1.6 M formic acid. The rest was dissolved in 0.5 mL of 6 M guanidine hydrochloride containing 2% β-mercaptoethanol and heated for 5 min in boiling water. This material was then chromatographed on Sephadex G-50 (1 × 46 cm) as described above.

Results

The kinetics of the reaction of the NAPS-chlorides with reduced glutathione was studied in 95% acetic acid. The reagent 2,4-NAPS-Cl reacted faster with reduced glutathione than 2,5-NAPS-Cl. The time required for half-maximal reaction was about 15 s for 2,4-NAPS-Cl compared to 45 s for 2,5-NAPS-Cl. The NAPS derivatives of glutathione were found to be homogeneous by TLC, and the introduction of a single NAPS group into the peptide was confirmed by elemental analysis. The absorption spectra of 2,4- and 2,5-NAPS-glutathione in acetic acid and 0.1 N NaOH are shown in Figure 1. The 2,4-NAPS derivative of glutathione has an absorption maximum at 385 nm (ε 3200) and strong absorption

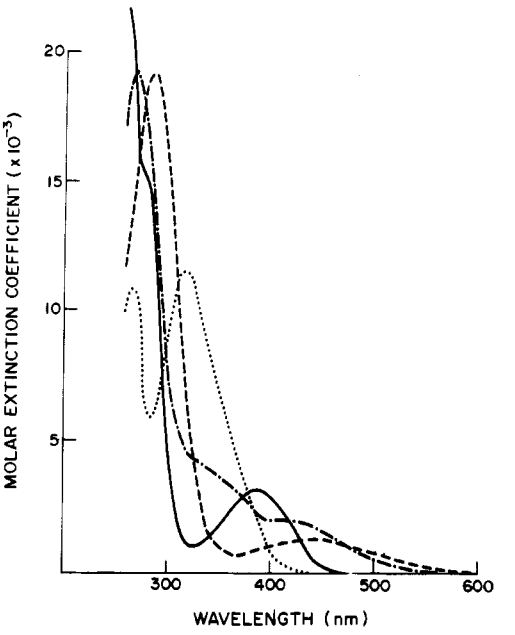

FIGURE 1: Absorption spectra of the NAPS derivatives of glutathione. 2,4-NAPS-glutathione in glacial acetic acid (—) and in 0.1 N NaOH (- - -); 2,5-NAPS-glutathione in glacial acetic acid (···) and in 0.1 N NaOH (-·-).

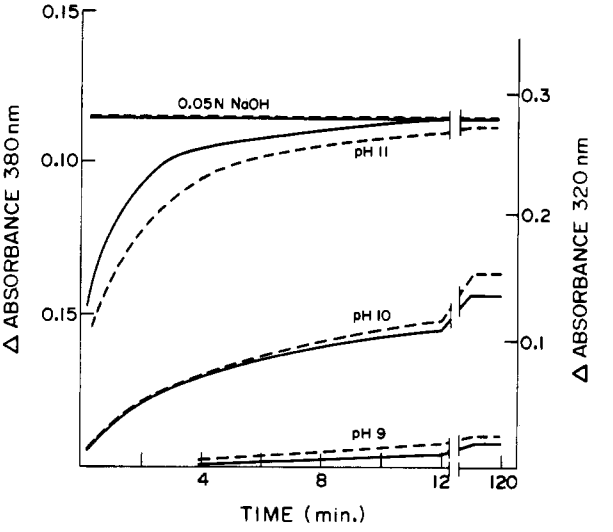

FIGURE 2: Stability of the disulfide bond in 2,4(5)-NAPS-glutathione. 2,4-NAPS-glutathione (solid lines) and 2,5-NAPS-glutathione (broken lines) were incubated at the indicated pH at room temperature, and the progress of cleavage was monitored as described under Materials and Methods.

below 300 nm in acetic acid. The 2,5-NAPS-glutathione has absorption maxima at 318 (ε 11 500) and 267 nm (ε 10 860). In 0.1 N NaOH, the absorption maxima of both derivatives shifted to longer wavelengths due to the release of 2-nitro-4-(5)-azidothiophenols by cleavage of the disulfide bonds. Alkali treatment of NAPS-glutathiones regenerated glutathione as shown by TLC on silica gel in solvent A.

The stability of NAPS-glutathione derivatives at various pH values was investigated by following the decrease in absorbance at 380 nm for the 2,4-NAPS derivative and at 320 nm for the 2,5-NAPS derivative of glutathione (Figure 2). Both were quite unstable above pH 11, and even at pH 10 only 50% of the derivatives remained intact after 2 h. However, no change in absorbance was observed at pH 8 or below after 24 h at 21 °C (data not shown).

The spectral changes produced upon photolysis of 2,4- and 2,5-NAPS-glutathione are shown in Figure 3. In the case of the 2,4-NAPS derivative, increases in absorbance at 450

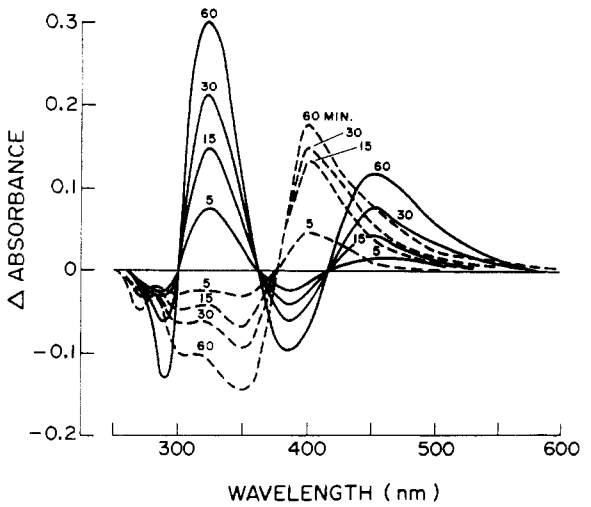

FIGURE 3: Difference spectra of photolyzed 2,4(5)-NAPS-glutathione. A solution of 1.58×10^{-4} M 2,5-NAPS-glutathione (solid lines) in acetic acid (optimal density at 366 nm of 0.38) or 2.35×10^{-4} M 2,5-NAPS-glutathione (broken lines) in acetic acid (optimal density of 366 nm of 1.18) was photolyzed at 10 °C as described previously (Muramoto & Ramachandran, 1980).

and 320 nm and decreases in absorbance at 385 and 290 nm were observed. 2,5-NAPS-glutathione showed an increase in absorbance at 350 nm upon photolysis. The kinetics of photolysis as well as the spectral changes of these NAPS-glutathione derivatives were found to be similar to the photoreactivities and spectral changes previously reported for the NAPS derivatives of *N*-acetyltryptophanamide (Muramoto & Ramachandran, 1980).

The photoreactive chlorides were also employed for the selective modification of [Trp(SH)^9]ACTH. The [Trp(SH)^9]ACTH was obtained by thiolysis of [2,4-dinitrophenylsulfenyl-Trp^9]ACTH as previously described (Canova-Davis & Ramachandran, 1980). For the thiolysis reaction, Wilchek & Miron (1972) used 0.1 M $NaHCO_3$. We found that a small amount of the [Trp(SH)^9]ACTH produced by thiolysis was oxidized to the dimer (R_f 0.31 in solvent B) during the manipulation of gel filtration to remove the salt. Therefore we conducted the thiolysis of [2,4-DNPS-Trp^9]ACTH in 0.1 M NH_4HCO_3 (pH 8.5). Under these conditions, no oxidation of [Trp(SH)^9]ACTH occurred. The NAPS derivatives of [Trp(SH)^9]ACTH were obtained in good yields.

The absorption spectra of [2,4-NAPSS-Trp^9]ACTH and [2,5-NAPSS-Trp^9]ACTH are shown in Figure 4. The spectrum of [2,4-NAPSS-Trp^9]ACTH exhibits a shoulder at 380 nm (ϵ 4000) and a maximum at 265 nm (ϵ 28 500). The spectrum of [2,5-NAPSS-Trp^9]ACTH has a maximum at 310 nm (ϵ 15 800). The molar extinction coefficients of the NAPS derivatives of [Trp(SH)^9]ACTH at wavelengths above 300 nm (which is due to absorption by the NAPS group) are very similar to the molar extinction coefficients of the model compounds (NAPS derivatives of glutathione), indicating that the peptide hormone was modified selectively at a single site. The photoreactivities of these derivatives were examined in 0.1 M phosphate buffer, pH 7.4. Both [2,4-NAPSS-Trp^9]ACTH and [2,5-NAPSS-Trp^9]ACTH were photolyzed more rapidly than the corresponding glutathione derivatrives (data not shown). The formation of covalently cross-linked aggregates of the ACTH derivatives was investigated by subjecting the products of photolysis to gel filtration under conditions capable of dissociating noncovalently bound aggregates. As shown in Figure 5, photolysis of both derivatives at neutral pH resulted in the formation of covalently linked dimers as well as polymers. When the photolyzed ACTH derivatives were treated

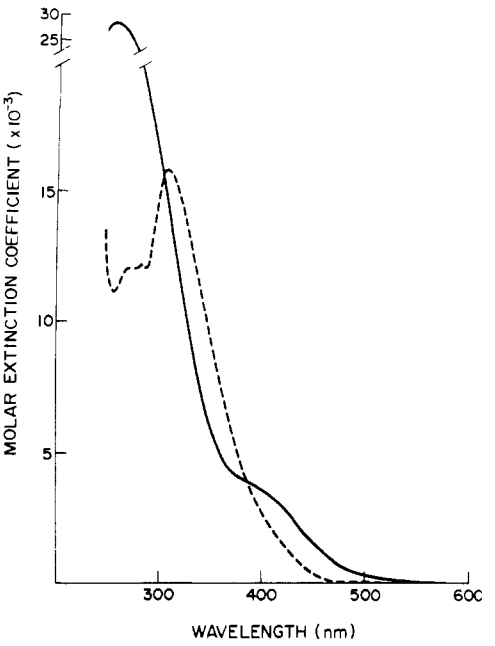

FIGURE 4: Absorption spectra of [2,4(5)-NAPSS-Trp^9]ACTH. The absorption spectra of [2,4-NAPSS-Trp^9]ACTH (solid line) and [2,5-NAPSS-Trp^9]ACTH (broken line) were measured in 2 M acetic acid.

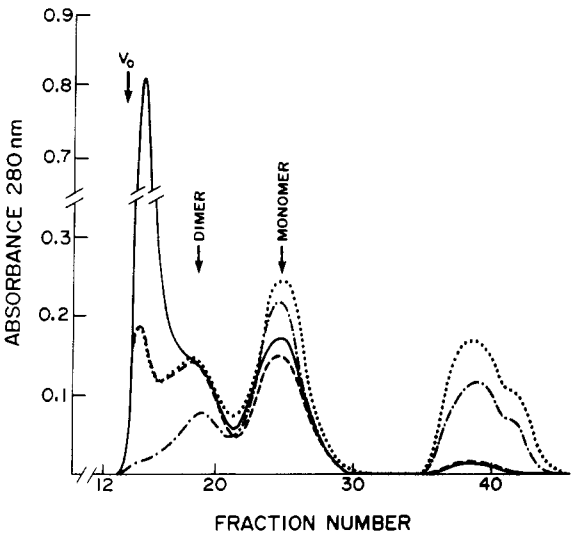

FIGURE 5: Gel filtration of photolyzed [2,4(5)-NAPSS-Trp^9]ACTH. The NAPS derivatives of [Trp(SH)^9]ACTH were photolyzed at pH 7.4 in 0.1 M phosphate buffer and the products fractionated on Sephadex G-50 in 1.6 M formic acid as described under Materials and Methods. The elution positions of the monomeric and dimeric photolysis products are indicated. Photolyzed [2,4-NAPSS-Trp^9]-ACTH before (—) and after (···) treatment with β-mercaptoethanol. Photolyzed [2,5-NAPSS-Trp^9]ACTH before (- - -) and after (-·-) treatment with β-mercaptoethanol.

with β-mercaptoethanol prior to gel filtration, the amount of polymers and dimers decreased, and the amount of monomeric ACTH product increased.

In an attempt to elucidate if there was a preferential site of interaction of the photogenerated nitrene, we isolated the photodimerized [NAPS-Trp^9] derivatives of ACTH as well as the monomeric products and determined the amino acid composition of acid hydrolysates (Table I). Compared to the unphotolyzed derivative, both the monomer and dimer of [2,4-NAPS-Trp^9]ACTH as well as [2,5-NAPS-Trp^9]ACTH showed a decrease in the amount of proline, valine, tyrosine, and phenylalanine. Partition chromatography of the monomeric photolysis product as previously described (Canova-

Table I: Amino Acid Composition of [2,4(5)-NAPS-Trp^9]ACTH before and after Photolysis[a]

	[2,4-NAPS-Trp^9]ACTH			[2,5-NAPS-Trp^9]ACTH		
		photolyzed			photolyzed	
amino acid	unphotolyzed	monomer	dimer	unphotolyzed	monomer	dimer
Asp-2[b]	1.9	2.1	2.1	1.9	2.1	2.1
Ser-2	1.7	1.7	1.6	1.7	1.8	2.1
Glu-5	5.0	5.0	5.0	5.0	5.0	5.0
Pro-4	4.2	3.6	3.4	4.2	3.8	3.2
Gly-3	3.2	2.9	2.8	3.2	3.0	3.1
Ala-3	3.1	2.7	3.0	3.1	2.9	3.0
Val-3	3.1	2.5	2.7	3.2	2.5	2.7
Met-1	0.9	0.8	0.8	1.0	1.0	0.7
Leu-2	2.1	2.3	1.9	2.1	2.1	1.9
Tyr-2	1.9	1.8	1.4	1.9	2.0	1.5
Phe-3	2.9	3.0	2.7	2.9	3.1	2.7
His-1	1.0	1.2	1.0	1.0	1.2	1.0
Lys-4	4.0	4.9	4.7	3.9	5.0	4.9
Arg-3	3.3	3.5	3.2	3.2	3.6	2.8

[a] [2,4(5)-NAPS-Trp^9]ACTH was photolyzed in 0.1 M phosphate buffer, pH 7.4, for 15 min, and the products were fractionated on Sephadex G-50 as shown in Figure 5. The monomeric and dimeric products were isolated, hydrolyzed in 5.7 N HCl at 110 °C for 22 h, and analyzed on a Beckman 119-C analyzer. [b] Theoretical values.

Davis & Ramachandran, 1976) resulted in a very broad peak, indicating the presence of closely related molecules which could not be resolved into homogeneous products in sufficient yield to permit unambiguous identification of the site of interaction of the nitrene.

Discussion

The results show that 2,4-NAPS-Cl and 2,5-NAPS-Cl are highly useful reagents for the introduction of a photoreactive group at thiol groups of peptides. These reagents cause rapid and quantitative modification of thiol groups of cysteine residues as well as thiol groups at the 2 position of the indole side chain of tryptophan residues. 2,4-NAPS-Cl appears to react faster than the isomeric reagent 2,5-NAPS-Cl. A similar difference in the reactivities of the two reagents was also observed in the modification of the indole moiety of tryptophan (Muramoto & Ramachandran, 1980). Although the reaction with the indole group is slower than that with the thiol group, the difference in the rates may not be sufficient to permit selective modification under acidic conditions. The reaction with the indole group proceeds much more slowly at higher pH. Peptides selectively modified at the thiol group may be prepared by performing the reaction at neutral pH and treating the product with acid to remove arylsulfenyl groups that may have reacted with amino groups. The use of these photoreactive reagents for selective modification of amino groups has also been investigated (K. Muramoto and J. Ramachandran, unpublished results).

The spectral characteristics of the NAPS groups attached to thiols are quite similar to those of NAPS groups attached to the indole group. This permits the use of radiation in the near-ultraviolet range (>350 nm) for photolysis, thus minimizing damage to the peptide or the biological component to which the peptide is bound.

The NAPS derivatives of glutathione are quite stable under neutral and acidic conditions but are readily cleaved to the parent thiol peptide at pH 10 or above. The disulfide bond between glutathione and the NAPS group is, of course, readily broken by reduction with β-mercaptoethanol (data not shown). Thus it is possible to remove the ligand peptide from its receptor under mild conditions after covalent attachment through photolabeling. The removal of the NAPS group from the indole by thiolysis was found to be incomplete (Muramoto & Ramachandran, 1981). Hence, we prepared derivatives of ACTH containing the photoreactive groups linked to the 2 position of indole through a disulfide bridge. [2,4-NAPSS-Trp^9]ACTH and [2,5-NAPSS-Trp^9]ACTH were obtained in good yield by reaction of [Trp(SH)^9]ACTH with 2,4-NAPS-Cl and 2,5-NAPS-Cl, respectively. The photoreactivities of these two compounds were significantly greater than those of the corresponding glutathione derivatives. A similar difference in the photoreactivities of the ACTH derivatives and the corresponding model compounds derived from *N*-acetyltryptophanamide has been noted previously (Muramoto & Ramachandran, 1980). These results suggest that the photoreactivities of the NAPS groups are sensitive to the environment and the efficiency of photolabeling is likely to be higher than what is observed with model compounds.

Photolysis of the NAPS derivatives of [Trp(SH)^9]ACTH at neutral pH resulted in the formation of covalently linked dimers and polymers (Figure 5). This is not surprising since ACTH is known to aggregate at neutral pH (Squire & Li, 1961). Under acidic conditions, very little dimer formation could be detected after photolysis (data not shown). This is consistent with the fact that the tendency to aggregate decreases with decreasing pH (Squire & Li, 1961).

In an attempt to delineate the region of the ACTH sequence involved in the interaction with the photoreactive group of the hormone, the monomeric and dimeric products of photolysis were isolated by gel filtration under conditions capable of dissociating noncovalent aggregates. Amino acid analysis showed that photolysis resulted in the partial loss of proline, valine, tyrosine, and phenylalanine (Table I), suggesting that these amino acid residues may be in the vicinity of the photoreactive group. Brunner & Richards (1980) have reproted that in gramicidin A tryptophan residues were preferentially attacked by nitrene and carbene. Both the monomeric and dimeric photolysis products of the [NAPS-Trp^9] derivatives of ACTH were found to be heterogeneous by partition chromatography. Therefore, it was not possible to identify the site(s) involved in covalent bond formation with the photogenerated nitrene. However, photoaffinity labeling can still be very useful in delineating the unique sites of interaction of ligands with macromolecules. The use of photoreactive derivatives of ACTH in elucidating the interaction of the hormone with bovine serum albumin is presented in the following paper in this issue. The results presented here indicate that these photoreactive arylsulfenyl chlorides may be useful for introducing a photoreactive group at the active sites of enzymes possessing a cysteine residue at the active site. Photolysis of the modified enzyme may then help to identify the amino acid residues in the active site.

Acknowledgments

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

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

Koji Muramoto and J. Ramachandran*

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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