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Synthesis and Characterization of a Heterobifunctional Photoaffinity Reagent for Modification of Tryptophan Residues and Its Application to the Preparation of a Photoreactive Glucagon Derivative†

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ABSTRACT: The synthesis of the heterobifunctional cross-linking reagent 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl) is described. This reagent can be used to specifically attach a photoactivatable nitrophenyl azide to tryptophan-containing polypeptides and proteins lacking sulfhydryl groups. The sulfenyl chloride group of NAPSCl reacts with the indole ring of tryptophan following second-order reaction kinetics in 50–100% acetic acid. The labeled product can be effectively photolyzed at wavelengths above 300 nm. The reaction of glucagon, a peptide hormone containing a single tryptophan residue at position 25 and no cysteine, with NAPSCl gave one major product, the photosensitive derivative glucagon-NAPS. The structure and properties of the purified derivative were established by amino acid analysis, absorption spectroscopy, and photolysis. Only the tryptophan residue of this derivative was modified. The photosensitive glucagon was shown to activate the adenylate cyclase of hepatocyte plasma membranes to the same extent as the native hormone at equimolar concentrations. Glucagon-NAPS could be radiolabeled by the lactoperoxidase-catalyzed iodination of the peptide. A glucagon-specific antibody bound both radiolabeled glucagon and glucagon-NAPS peptides. The covalent labeling of protein molecules with radiolabeled glucagon-NAPS peptide upon photolysis was demonstrated. Glucagon-NAPS can be used as an effective photoaffinity probe for labeling the glucagon receptor site in plasma membranes of target cells.

The technique of photoaffinity labeling with aryl azide derivatives and the criteria and advantages over conventional affinity labeling have been discussed (Knowles, 1972; Bayley & Knowles, 1977). One of the most important areas for the use of photoaffinity labeling has been the labeling of biological receptor sites in a variety of complex systems. Some examples are the photoaffinity labeling of putative receptors of acetylcholine (Witzemann & Raftery, 1977; Hucho et al., 1976), concanavalin A of human erythrocyte membrane (Ji, 1977), a peptide secretagogue in the exocrine pancreas (Galardy & Jamieson, 1977), cAMP[1] cytosolic proteins of various tissues (Walter et al., 1977), the antidiuretic hormone (Goodman et al., 1978), insulin (Wisher et al., 1978), and glucagon (Bregman & Levy, 1977). The advantage over other types of affinity labels in such systems is that the behavior and kinetics of ligand–receptor interactions can be studied in situ since aryl azides are chemically inert under physiological conditions in the dark and are activated only upon photolysis (Bayley & Knowles, 1977). Furthermore, equilibrium and maximal occupancy of the receptor can be ensured before photolysis, and upon photolysis the generated nitrene intermediate has a wide range of reactivity and is able to attack even inert C–H bonds. In contrast to the required nonselective labeling of the receptor, the labeling of the ligand with the photoactivatable group must be selective and specific and must not alter its physiological activity. In this report the synthesis and characterization of a sulfenyl halide photoaffinity reagent 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl) is presented. NAPSCl is a selective and specific reagent for tryptophan and tryptophan-containing polypeptides and proteins in accordance with the reactivity of similar nitrosulfenyl halides (Scoffone et al., 1968). Irradiation of the reagent with visible light affects photolysis of the azide moiety of the reagent.

NAPSCl was used to label the peptide hormone glucagon which has a single tryptophan residue at position 25. The glucagon-NAPS derivative has the potential for specific receptor photolabeling of plasma membrane components since, as expected from other tryptophan-modified glucagon ana-

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[1] Abbreviations used: NMR, nuclear magnetic resonance; IR, infrared; NPSCl, 2-nitrophenylsulfenyl chloride; DNPSCl, 2,4-dinitrophenyl-1,5-disulfenyl chloride; NAPSCl, 2-nitro-4-azidophenylsulfenyl chloride; glucagon-NPS, glucagon-2-nitrophenylsulfenyl; $(glucagon)_2$DNPS, $(glucagon)_2$-2,4-dinitrophenylsulfenyl; NAP-glucagon, (4-azido-2-nitrophenyl)glucagon; glucagon-NAPS, glucagon-2-nitro-4-azidophenylsulfenyl; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; GTP, guanosine 5′-triphosphate; EGTA, ethylene glycol bis(*p*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; KIU, Kallikrein International Units.

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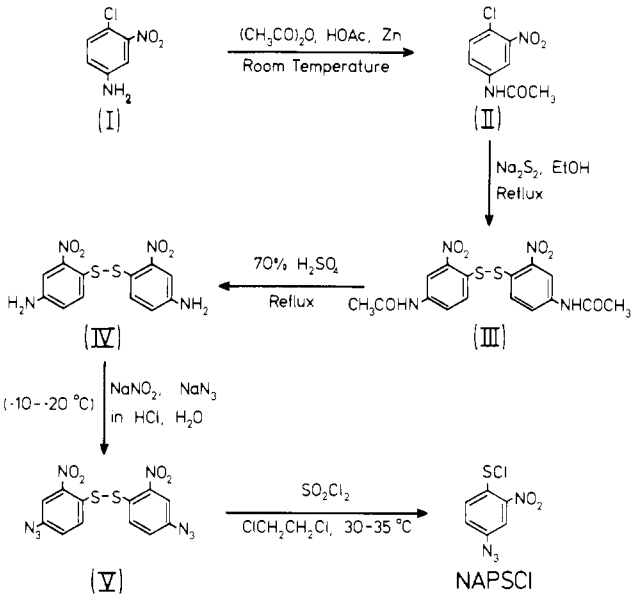
Scheme I



logues (Epand & Cote, 1976), it activates adenylate cyclase to the same extent as the native hormone, and it can covalently label proximal macromolecules upon photolysis.

Experimental Procedures

Materials

4-Chloro-3-nitroaniline was purchased from Aldrich Chemical Co.; sulfuryl chloride was from Eastman; potassium phthalate was from Matheson Coleman and Bell; bovine serum albumin (fraction V), Alumina WN-3, Dowex 50W-X8 (200–400 mesh), all amino acids, 98–100% grade creatine phosphate, and rabbit muscle type I creatine phosphokinase were from Sigma Chemical Co.; [2,8-^{3}H]ATP (34.8 Ci/mmol), [8-^{14}C]cAMP (53.1 mCi/mmol), and Na^{125}I (~17 Ci/mg) were from New England Nuclear; lactoperoxidase (260 units/mg, OD_{412}/OD_{280} = 0.74) and Trasylol (10 000 KIU/mL) were from Boehringer; protein A–Sepharose CL-4B, Sephadex G-75, and Sephacryl S-300 were from Pharmacia; bovine–porcine glucagon was purchased from Elanco Corp. The purity of the product was evaluated by partition chromatography (Figure 2). All other reagents used were of the highest purity available commercially.

Methods

The synthesis of 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl) reagent was accomplished by a series of reactions starting from 4-chloro-3-nitroaniline as shown in Scheme I.

Synthesis of 4-Chloro-3-nitroacetanilide (II). II was prepared from 4-chloro-3-nitroaniline (I) by applying the procedure for acetylation of aromatic amines (Vogel, 1956a). The product was recrystallized (twice) from 95% ethanol, giving pale yellow needle-shaped crystals (yield >76%) [mp 148–149 °C (lit. 150 °C); Weast (1972)]. This product as well as those of subsequent reactions was characterized by proton NMR, IR, and mass spectra. In all cases the results agreed with those expected for the reported compound, but for brevity they are not included in this report.

Synthesis of 2,2'-Dinitro-4,4'-diacetamidodiphenyl Disulfide (III). III was synthesized from II by a method analogous to that used to prepare bis(*o*-nitrophenyl) disulfide (Bogert & Stull, 1941). The product was recrystallized from dimethylformamide (yield >50%). The orange needle-shaped crystals decomposed above 200 °C.

Synthesis of 2,2'-Dinitro-4,4'-diaminodiphenyl Disulfide (IV). IV was synthesized by adopting the method for deacetylation of derivatives of aromatic amines (Vogel, 1956b). The reddish flaky crystals obtained (yield >79%) decomposed above 150 °C.

Synthesis of 2,2'-Dinitro-4,4'-diazidodiphenyl Disulfide (V). V was synthesized by reacting the diazonium salt of IV with hydrazoic acid according to the procedure for phenyl azide synthesis (Fleet et al., 1972). The product was recrystallized from dimethylformamide (yield >86%). The yellow needles obtained decomposed above 130 °C.

Synthesis of 2-Nitro-4-azidophenylsulfenyl Chloride (NAPSCl). NAPSCl was synthesized by chlorolysis of the disulfide (Kühle, 1973). V (3.6 g) in 1,2-dichloroethane (25 mL) was heated with stirring to 30–35 °C. Sulfuryl chloride (10 mL) was then slowly added until all the disulfide had dissolved. The solvent was removed under vacuum and the product recrystallized from ethyl acetate (yield >96%). Yellow needle-shaped crystals were obtained (mp 75–78 °C). Anal. Calcd for $C_6H_3N_4O_2SCl$: C, 31.24; H, 1.31; N, 24.24; S, 13.90; Cl, 15.37. Found: C, 31.35; H, 1.47; N, 24.19; S, 13.61; Cl, 15.16. NAPSCl was stored at −20 °C in the dark.

Reaction of NAPSCl with Amino Acids. A standard amino acid mixture containing 2.5 μmol/mL of all common amino acids was left to react with a 100% excess of NAPSCl in glacial acetic acid, in the dark at room temperature. After 2 h the reaction was stopped by adding 5 mL of water, and the precipitated unreacted reagent was removed by centrifugation. The supernatant was dried under vacuum; the amino acid mixture was redissolved in 1 mL of citrate buffer, pH 2.2, and an amino acid analysis was run on a Beckman 120C analyzer. The amino acid mixture was also exposed to glacial acetic acid in the absence of NAPSCl as a control.

NAPSCl was also incubated under similar conditions with either methionine, tyrosine, phenylalanine, or histidine for 30 min. Samples taken at regular time intervals after precipitation of the unreacted reagent were chromatographed on Whatman No. 1 paper using butanol–acetic acid–water (20:5:9 v/v) as the solvent. R_f values were compared with controls after spraying with ninhydrin reagent.

The rate of reaction of NAPSCl with tryptophan was determined by titration of the unreacted NAPSCl using the Orr & Kharash (1953) method as modified by Scoffone et al. (1968) or by fluorescence measurements. Quantitative estimation of the unreacted sulfenyl halide involved titrating thiosulfate with iodine quantitatively released by the sulfenyl halide from potassium iodide. Titration had to be carried out in the light, but the tryptophan–NAPSCl reaction solution was kept in the dark. For fluorescence measurements, aliquots of the reaction solution were removed at regular time intervals, and, after precipitating the unreacted reagent with water, the decrease in tryptophan fluorescence emission of the supernatant was measured with a Perkin-Elmer MPF-44 fluorometer with an excitation wavelength of 295 nm.

Reaction of Glucagon with NAPSCl. Glucagon-NAPS was prepared in the dark according to the method of Veronese et al. (1970) with the exception that the reaction was carried out with a 2:1 molar ratio of reagent to protein, to avoid nonspecific labeling. Unreacted reagent was removed by gel filtation through a Sephadex G-25 (fine) column (Veronese et al., 1970). Fractions were pooled and lyophilized (yield >95%), and the product was stored at 4 °C, in the dark, and in a desiccator.

Purification and Characterization of Glucagon-NAPS. Glucagon-NAPS was purified by partition chromatography

according to Hruby & Grosinsky (1971) by using the butanol–ethanol–benzene–0.2 N NH_4OH (5:2:1:8 v/v) solvent system, pH 9.4. The elution pattern was monitored by the Lowry et al. (1951) protein determination with bovine serum albumin as a standard and by absorption measurements at λ_{max} = 395 nm.

The purity of the glucagon-NAPS derivative was also checked by using 10% acrylamide gel electrophoresis according to Davis (1964). Gels were stained with Brilliant Blue-G according to Bromer et al. (1971). The purified glucagon-NAPS derivative was hydrolyzed in vacuo with 6 N HCl for 22 h at 110 °C, and the hydrolysate was subjected to an amino acid analysis.

Photolysis of NAPSCl and Glucagon-NAPS. A 0.3–0.4 mM solution of NAPSCl in glacial acetic acid was photolyzed for up to 1 h in a thermally regulated jacketed cell, 10 cm away from the light source (high-pressure xenon lamp, XBO, 150 W). The solution was bubbled with water-saturated N_2 gas 5 min prior to photolysis, and water-saturated gas was blown through the solution during photolysis at a rapid enough rate to ensure mixing of the solution. The circulating water bath contained ~3 mm thick 0.01% (v/v) potassium phthalate as a filter for ultraviolet light. The temperature of the cell, monitored with a thermistor, was maintained at 25 °C. At recorded time intervals, aliquots were removed and their absorbances at the λ_{max} of 420 nm were recorded. The aliquots were then dried under vacuum. IR spectra of these samples dissolved in chloroform were subsequently recorded.

Glucagon or glucagon-NAPS solutions (10 μM) in glacial acetic acid were photolyzed for 8 min under the same conditions as NAPSCl. Photolysis was monitored by absorption measurements at λ_{max} = 395 nm.

[^{125}I]Glucagon or [^{125}I]glucagon-NAPS in 0.2 M glycine, pH 6.5–8.8, was photolyzed under the above conditions with the addition of a glass filter (Corning CS No. 7–51, 4.9–5.1 mm) fitted between the light source and the phthalate filter. Photolysis was carried out for 15–20 min. The integrity of the photolyzed hormone derivatives was checked by gel filtration of the denatured peptides in a Sephadex G-75 column (50 × 0.5 cm) with 0.2 M glycine, pH 2.6, and 9 M urea, as the eluant. The elution profile was monitored by measuring the radioactivity of the fractions collected with a well-type γ counter (Beckman Gamma-300). Nonphotolyzed samples were run under similar conditions as controls.

Photochemical Properties. Various concentrations (10^{-5}–10^{-4} M) of NAPSCl in glacial acetic acid were prepared in duplicate. One set was kept in the dark, and the other was exposed to ordinary fluorescent room lighting. Decomposition of the azide derivative with time was monitored by changes in OD_{420}. The percent change in OD_{420} was compared with those of similar sets photolyzed immediately and 48 h (kept in the dark) after preparation.

2,2′-Dinitro-4,4′-diazidodiphenyl disulfide (10^{-4} M) was photolyzed for 15 min under the same conditions as NAPSCl, and the products of photolysis were analyzed by thin-layer chromatography on silica gel plates with a fluorescent indicator in methylene chloride–acetic acid (9:1 v/v; solvent A) or ethyl acetate (solvent B), at room temperature (23 °C). The spots were visualized under short UV light. 2,2′-Dinitro-4,4′-diaminodiphenyl disulfide was used as a standard for the detection of any diradical formation of nitrene upon irradiation which is likely to result in the formation of a primary amine (Hanstein et al., 1979).

Iodination of Glucagon and Glucagon-NAPS. Glucagon and the glucagon-NAPS derivative were iodinated by the lactoperoxidase method at pH 10.0 in the presence of propylene glycol (Von Schenk & Jeppsson, 1977). The amount of H_2O_2 was equivalent to that of Na^{125}I (0.3 g-atom of ^{125}I/mol of glucagon). A 2.5-μg quantity of lactoperoxidase was used. The reaction time was 60 s. The reaction was stopped by the addition of 1.0 mL of 0.1 M phosphate buffer, pH 7.5, containing 0.05% NaN_3. The radioiodinated peptide was purified from unreacted ^{125}I by the talc absorption method (Goldstein & Bletcher, 1976). The hormone was eluted from talc with 50% ethanol and lyophilized immediately. The lyophilized material was redissolved in 1 mM NH_4HCO_3, pH 9.5, and applied to a DEAE-cellulose column (1 × 17 cm, DE-52, Whatman) swollen in the same medium. The monoiodopeptide derivative was eluted according to the method described by Desbuquois (1975). The tubes containing the monoiodopeptide were pooled and dialyzed against 100 mM NH_4HCO_3, pH 7.9, at 4 °C. After removal of the NH_4HCO_3 by lyophilization, the monoiodopeptide was stored in propanol–water (1:1) solution at −20 °C (sp act. ~2.47 μCi/pmol).

Photoaffinity Labeling of Bovine Serum Albumin and a Glucagon-Specific Antibody by [^{125}I]Glucagon-NAPS. [^{125}]Glucagon or [^{125}I]glucagon-NAPS (10 000–20 000 cpm, 1.8 μCi/pmol) was incubated in 5 mL of a solution of 0.2 M glycine, pH 6.5–7.0, 2.5 mg/mL BSA, and 750 KIU/mL Trasylol, in the dark for 10 min at room temperature. The samples were then photolyzed as described above and subsequently lyophilized. The protein was denatured with 0.2 M glycine, pH 2.6, and 9 M urea, and the noncovalently bound radiolabeled hormone was removed through a Sephadex G-75 column as previously described. The elution profile was monitored by UV absorption at 278 nm and by counting the fractions collected. Nonphotolyzed samples similarly treated were also run in the dark.

Equivalent amounts of radiolabeled peptides were also incubated with 1:10 diluted glucagon-specific antiserum or normal rabbit serum. These sera prepared according to the method of Tager et al. (1977) were generously donated to us by Dr. J. Gauldie, McMaster University. Their affinity for glucagon was measured by Dr. N. Track, University of Toronto, by radioimmunoassay and found suitable for this purpose.

The total volume of samples was 0.25 mL containing 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10.0% glycerol, 2.0 mg/mL BSA, and 750 KIU/mL Trasylol. After incubation at 4 °C for 72 h in the dark, the samples were diluted 10 times with the same buffer, the pH was adjusted to 6.5–7.0 with dilute HCl, and they were photolyzed under the above conditions for 20 min.

After photolysis the radiolabeled IgG was purified in a Sephacryl S-300 column (80 × 2 cm) by using the same incubation buffer as eluant. The pooled IgG fractions were denatured with 9 M urea after lyophilization and the noncovalently bound radiolabeled hormone was removed by gel filtration through a Sephadex G-75 column as described above. Nonphotolyzed samples similarly treated in the dark were used as controls.

Radioimmunoprecipitation of [^{125}I]Glucagon and [^{125}I]-Glucagon-NAPS. [^{125}I]Glucagon and [^{125}I]glucagon-NAPS (10 000–20 000 cpm, 1.8 μCi/pmol) were incubated with glucagon-specific antiserum and normal serum and subsequently photolyzed as described above. As controls, other samples were similarly treated but not photolyzed. After photolysis, 20 μL of settled protein A–Sepharose CL-4B swollen in the same buffer was added to each sample. The suspensions were shaken for 24 h at 4 °C. The immunopre-

cipitate protein A–Sepharose complex was then washed once with ice-cold buffer containing 250 mM LiCl, 50 mM Tris-HCl, pH 9.0, and 1% (v/v) 2-mercaptoethanol and 3 times with 125 mM LiCl, 25 mM Tris-HCl, pH 9.0, and 0.5% (v/v) 2-mercaptoethanol according to the method of Schauffhausen (1978). The precipitate was solubilized with 60 mM Tris-HCl, pH 6.8, 6% (w/v) sodium dodecyl sulfate, 10% glycerol, and 10% (v/v) 2-mercaptoethanol and counted.

Spectrophotometric Studies. All absorption measurements and spectra were recorded with a Cary 118 spectrophotometer.

Membrane Purification. Partially purified rat liver plasma membrane was prepared by using male Wistar rats (150–200 g). In a typical preparation, the rat was decapitated and the liver was perfused with 50 mL of ice-cold 1 mM $NaHCO_3$, pH 7.4, through the inferior vena cava to remove the blood. Liver plasma membrane was subsequently prepared according to the modified Neville procedure (Pohl et al., 1971). During homogenization 20 strokes were employed instead of 8 and the partially purified membrane was washed 4 times with 1 mM $NaHCO_3$, pH 7.4, according to Pilkis et al. (1974). Membrane fractions (1 mL) were frozen in liquid N_2 and stored at −70 °C.

The purity of the final preparation was evaluated by the use of marker enzymes. 5′-Nucleotidase (EC 3.1.3.5) was measured by the method of Bodansky & Schwartz (1963) and glucose 6-phosphatase (EC 3.1.3.9) by the method of Toda et al. (1975). The enzymes were assayed in 1 mL of incubation medium for 15 min at 37 °C. Reactions were stopped by the addition of 1 mL of 10% trichloroacetic acid. Liberated inorganic phosphate was measured by the method of Fiske & Subba Row (1925). Succinate–cytochrome *c* reductase was measured by the method of King (1967).

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the technique of Krishna et al. (1968) using [^{3}H]ATP as a substrate and [^{14}C]cAMP as a tracer. The final assay medium contained 30 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM mercaptoethanol, 0.2 mM cAMP, 10 μM GTP, 0.56 mM [^{3}H]ATP, 5 mM creatine phosphate, 0.64 mg/mL creatine phosphokinase (50–60 units/mL), 1 mM aminophylline, and 4 mg/mL bovine serum albumin in a final volume of 100 μL. Glucagon, glucagon-NAPS, or 20 mM NaF in 30 mM Tris-HCl containing 1 mg/mL BSA, pH 7.6, was included as indicated. After a 1-min incubation of the membrane (10–30 μg) at 30 °C, the reaction was initiated by the addition of [^{3}H]ATP (2.5 μCi, 100 dpm/pmol). The reaction was stopped by the addition of 150 μL of 1 N $HClO_4$. An additional 265 μL of water and 10 μL of [^{14}C]cAMP tracer (3 000–10 000 cpm) were added, and cAMP was isolated by reversing the procedure reported by White & Karr (1978) using first alumina columns (Alumina WN-3, Brockman affinity II–III) and then by Dowex 50 columns (Dowex 50W-X8, 200–400 mesh).

Results

Reaction of NAPSCl with Tryptophan. The specificity of the sulfenyl chloride moiety of the synthesized NAPSCl was established by reaction of the reagent with a standard mixture of amino acids (Table I), as well as with glucagon (Table II). The results indicated that tryptophan and, to a small extent, methionine are the only amino acid residues, in the absence of cysteine, to undergo reaction. Methionine may undergo oxidation rather than reaction with the sulfenyl chloride moiety. Paper chromatography of a NAPSCl reaction mixture with tyrosine, phenylalanine, methionine, and histidine gave R_f values indistinguishable from those of the unmodified amino acids, indicating that the electrophilic NAPSCl reagent remains largely unreactive toward those amino acids.

Table I: Recovery of Amino Acids Reacted with 2-Nitro-4-azidophenylsulfenyl Chloride[a]

amino acid	fraction recovered		amino acid	fraction recovered	
	exptl[b]	control[c]		exptl[b]	control[c]
Asp	1.1	1.1	Met	0.9	1.0
Thr	1.1	1.0	Ile	1.0	1.0
Ser	1.2	1.0	Leu	1.0	1.0
Glu	1.0	1.0	Tyr	1.0	1.0
Pro	1.1	1.2	Phe	1.0	1.0
Gly	1.1	1.0	His	1.0	1.0
Ala	1.1	1.0	Lys	1.0	1.0
Cys[d]	0.9	1.0	Arg	1.0	1.0
Val	1.1	1.0	Trp	0.0	1.2

[a] A standard amino acid mixture (2.5 μmol/mL) of all common amino acids was reacted in duplicate with a 100% molar excess of NAPSCl in glacial acetic acid in the dark at room temperature for 2 h. [b] Average values of duplicate determinations. [c] The amino acid mixture was exposed to glacial acetic acid in the absence of NAPSCl. [d] Half-cystine.

Table II: Amino Acid Composition of Glucagon-NAPS Residues per Mole of Peptide

amino acid	found[a]	expected[b]	amino acid	found[a]	expected[b]
Asp	4.3	4	Leu	2.0	2
Thr	3.0	3	Tyr	1.9	2
Ser	3.6	4	Phe	1.9	2
Glu	3.2	3	His	0.9	1
Gly	1.3	1	Lys	1.0	1
Ala	1.1	1	Arg	2.0	2
Val	0.9	1	Trp[c]	0.0	I
Met	0.8	1			

[a] Peptide purified by partition chromatography. Values given are for 22-h hydrolysis at 110 °C in 6 N HCl calculated on the basis of 28 residues. [b] Based on known amino acid composition (Bromer et al., 1957). Aspartic and glutamic values include production from asparagine and glutamine, respectively. [c] Destroyed on acid hydrolysis, not determined.

The kinetics of the reaction with tryptophan was followed by determining the rate of disappearance of the sulfenyl chloride via the reaction $2Ar\text{–}S\text{–}Cl + 2I^- \rightarrow Ar\text{–}S\text{–}S\text{–}Ar + I_2 + 2Cl^-$. By titration of the free iodine, the percent of reacted tryptophan was calculated on the basis of the consumption of the reagent. Data were found to conform to a second-order reaction (Figure 1). Similar results were obtained by measuring the fluorescence of the remaining tryptophan (Figure 1). The rate constant of the reaction was calculated on the basis of the titration data only since fluorescence data were more subject to experimental error. In both cases, 50% of tryptophan had reacted within 5–6 min. NAPSCl, therefore, reacted with tryptophan ~8 times faster than NPSCl (Scoffone et al., 1968). Faster reaction rates of sulfenyl chlorides have been reported by Scoffone et al. (1968) with tryptophan-containing peptides as compared to the free amino acid.

Synthesis and Characterization of Glucagon-NAPS. Reaction of NAPSCl with glucagon in a 2:1 molar ratio resulted in quantitative labeling of tryptophan-25. Only one major species of glucagon-NAPS was observed by both partition chromatography (Figure 2) and polyacrylamide gel electrophoresis. The small contaminant (<5%) observed in both native and modified glucagon could be due to deamidated glucagon since it only differed by one charge, as was indicated by gel electrophoresis (data not shown).

Fluorescence studies of the modified derivative in 7 M guanidine hydrochloride and 10 mM glycine, pH 8.1, at 350

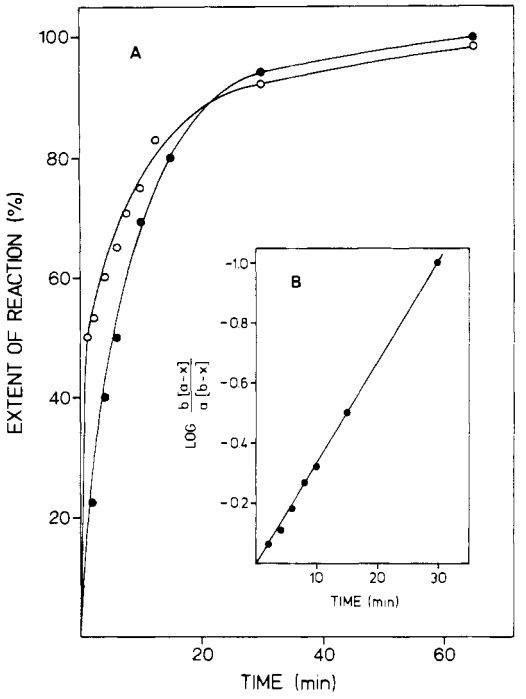


FIGURE 1: Rates of reaction of 2-nitro-4-azidophenylsulfenyl chloride with tryptophan in a 2:1 ratio in glacial acetic acid. (A) Percent loss of tryptophan fluorescence with time (○); percent of reacted tryptophan with time calculated on the basis of the remaining unreactive reagent titrated with free iodine (●). (B) Linear plot of the kinetic equation for a second-order reaction from the titration data of the NAPSCl–tryptophan reaction: a, initial concentration of NAPSCl; b, initial concentration of tryptophan; $a - x$ and $b - x$, concentrations remaining at a given time.

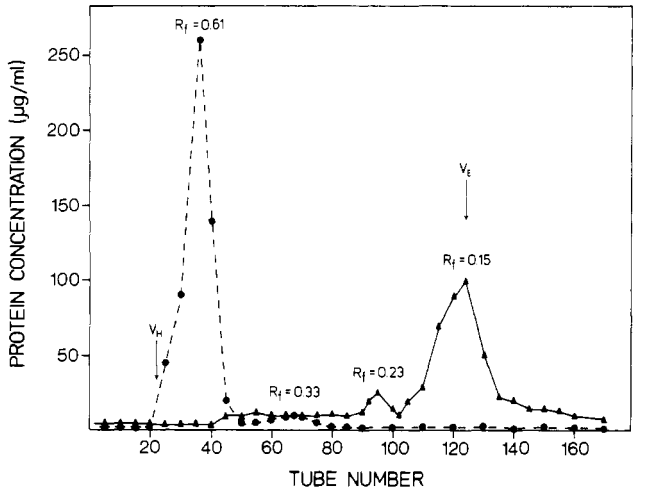


FIGURE 2: Partition chromatography of glucagon and glucagon-NAPS derivative. The solvent system used was butanol–ethanol–benzene–0.2 N NH_4OH (5:2:1:8 v/v), pH 9.4. Samples were applied to a Sephadex G-25 (fine) column (1.5 × 90 cm), and 2.0-mL fractions were collected at a rate of 8–10 mL/h. V_H = hold-up volume; V_E = elution volume; $R_f = V_H/V_E$. Glucagon (▲); glucagon-NAPS (●).

nm where glucagon fluoresces with an excitation wavelength of 295 nm indicated that there is $<(4.3 \pm 0.1) \times 10^{-2}$% contamination with unmodified glucagon calculated on the basis of the detection limits of the technique used (data not shown).

Photoreactivity of NAPSCl and Glucagon-NAPS. The photoreaction of the azide moiety of NAPSCl was followed by changes in OD at λ_{max} = 420 nm upon irradiation with light (Figure 3). These data and the disappearance of the IR band at 2120 cm^{-1} (data not shown) indicated that photolysis was completed within 6 min. When the glass filter no. 7-51 was added, the photolysis was completed within 25 min (data not

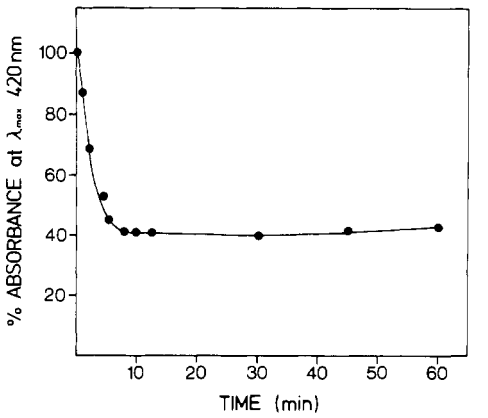


FIGURE 3: Rate of photolysis of 2-nitro-4-azidophenylsulfenyl chloride. A 0.3–0.4 mM solution of NAPSCl in glacial acetic acid, at 25 °C, under N_2 was irradiated with light at wavelengths >300 nm. At designated time intervals, the irradiation was stopped, 1-mL aliquots were removed, and their absorbances at λ_{max} 420 nm were measured. The results are the average of duplicate determinations.

Table III: Effect of Room Lighting on the Stability of 2-Nitro-4-azidophenylsulfenyl Chloride[a]

time (h)	% OD_{420} remaining	
	dark	light[b]
0	100	100
3	96.7	94.8
15	96.0	73.0
26	91.7	59.4
60	84.7	37.8
photolyzed[c] at zero time, % OD_{420} = 45		
photolyzed[c] 48 h after being in the dark, % OD_{420} = 20		

[a] 10^{-5}–10^{-6} M solutions of NAPS reagent in glacial acetic acid were prepared in the dark and their absorbance at 420 nm was measured at various time intervals. [b] Ordinary fluorescent light in the absence of any aberrant light. [c] Photolyzed refers to exposure of the solution for 10 min at 25 °C under N_2 using a 150-W xenon arc lamp at wavelengths above 300 nm.

shown). The percent change in OD_{420} was also used to determine the stability of NAPSCl under ordinary fluorescent room light in the absence of any daylight (Table III). For solutions kept in the dark, there was only a 15% change in OD_{420} after 60 h, while there was more than a 50% change for those exposed to light. Over a 50% decrease in OD_{420} was also observed for the solutions photolyzed immediately after preparation, while there was a >70% decrease for those kept in the dark and photolyzed 48 h later, indicating that some decomposition of the reagent may take place upon standing in acidic solutions at room temperatures over long periods of time. The 5.2% change after 3 h for the solutions exposed to light indicated that the azide is not very susceptible to photolysis under laboratory light conditions over short time periods.

Thin-layer chromatography of the irradiation product mixture of 2,2′-dinitro-4,4′-diazidodiphenyl disulfide indicated that no product comigrated with the authentic sample of 2,2′-dinitro-4,4′-diaminodiphenyl disulfide (within the detection limits of the technique used). Thus the azide produces predominantly singlet nitrene upon irradiation and can be used successfully for photoaffinity labeling.

Glucagon-NAPS and the photolyzed derivative gave characteristic absorption spectra and are compared to that of glucagon (Figure 4). The decrease in OD_{395} was characteristic of the photoreaction of the aryl azide moiety. The absorption maxima and the molar extinction coefficients of the sulfenylated tryptophan with the monofunctional reagent 3-nitrophenylsulfenyl chloride (NPSCl) and with NAPSCl, as well

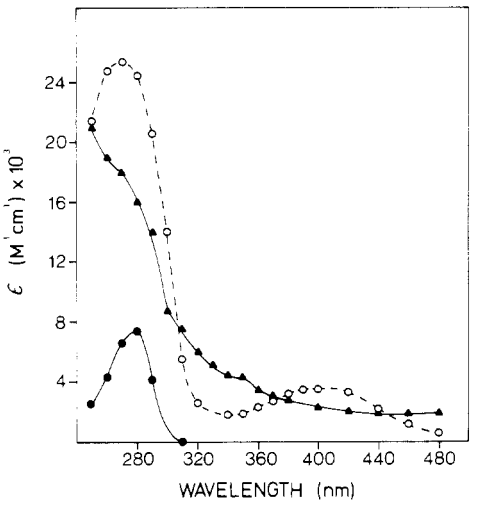


FIGURE 4: Absorption spectra of glucagon (●), glucagon-NAPS (○), and photolyzed glucagon-NAPS (▲) in glacial acetic acid. Photolysis of glucagon-NAPS solution (10^{-6} M) was at 25 °C under N_2 for 8 min.

Table IV: Absorption Characteristics of Compounds Used in This Work

compound	solvent	λ_{max} (nm)	$\epsilon \times 10^{-3}$ (M^{-1} cm^{-1})
NPSCl	HOAc	391	3.20
NAPSCl	HOAc	420	2.95
NAPSCl[a]	HOAc	420	1.28
Trp-NPS	H_2O	361	1.88
Trp-NAPS	H_2O	395	4.55
Trp-NAPS[a]	H_2O	395	3.25
glucagon[b]	HOAc	280	8.31
glucagon-NAPS	HOAc	395	3.60
glucagon-NAPS	HOAc	275	24.90
glucagon-NAPS[a]	HOAc	395	1.10

[a] Photolyzed at 25 °C under N_2 for 8 min. [b] Spectrum unaltered by photolysis.

as with glucagon-NAPS and native glucagon, both before and after photolysis are compared in Table IV.

Iodination by Lactoperoxidase at pH 10.0. The glucagon-NAPS derivative was effectively radiolabeled; however, the extent of iodination was ~30% lower than that observed for the native hormone under similar conditions. The ion exchange purified monoiodinated hormone derivatives were used in all photolabeling experiments. Storage at −20 °C in a 1:1 (v/v) propanol–water solution resulted in ~14% high molecular weight aggregates and ~5.0% radiolysis products after 60 days as determined by gel filtration through a P-10 column (50 × 0.5 cm) with 154 mM NaCl, 3.2 mM EGTA, 0.01% NaN_3, 0.1% BSA, and 750 KIU/mL Trasylol, pH 7.4, buffer as the eluant.

Stability of the Radiolabeled Hormone to Photolysis. Iodination of the tyrosine residues of the hormone results in an increase in their extinction coefficient, a shift of the absorption maximum to the red, and a lowering of the p*K* (Edelhoch, 1962). This latter effect results in the products of photolysis being pH dependent in the region pH 7–10 with a loss of covalently bound ^{125}I occurring at higher pH, presumably as a result of direct photolysis of iodotyrosinate. Only 16% of [^{125}I]glucagon was recovered in the fractions where glucagon elutes when photolysis was carried out at pH 8.8. On the other hand, >85% was recovered in these fractions when the radiolabeled hormone was photolyzed at pH 6.5–7.0. As a result, photolysis of the radiolabeled hormone derivatives was carried out at neutral pH. Under these conditions there is no migration of ^{125}I, nor is there any polymerization of the glucagon derivative.

Photoaffinity Labeling of Bovine Serum Albumin (BSA) and Anti-Glucagon-IgG. The ability of the monoiodinated glucagon-NAPS derivative to be covalently cross-linked to other molecules upon photolysis was tested by using BSA. The percent radiolabeling of BSA measured under denaturing conditions was dependent on the concentration of BSA resulting in 20–25% cross-linking at 2.5 mg/mL after photolysis for 2 times the half-life of the photoaffinity reagent. Nonphotolyzed samples as well as those photolyzed in the presence of [^{125}I]glucagon showed only 2.0–2.5% radioactivity associated with BSA. This radioactivity was attributed to high molecular weight aggregates which spontaneously formed the radiolabeled hormone peptides presumably as a result of radiolysis damage.

Protein A radioimmunoprecipitation as well as gel filtration of the [^{125}I]glucagon or [^{125}I]glucagon-NAPS–glucagon antibody complex showed that 75–85% of the radiolabeled hormone was precipitated with the glucagon-specific antiserum, while only 5–10% was immunoprecipitated with the normal serum. Specific immunoprecipitation was ~10% lower in the samples photolyzed prior to incubation with protein A or gel filtration.

Covalent labeling of the anti-glucagon-IgG molecule measured by G-75 gel filtration with urea was 40% of the immunoprecipitated material in the photolyzed sample compared to 17% in the nonphotolyzed control. Similarly, 15–20% of the radioactivity which was associated nonspecifically with the IgG fraction of normal serum was recovered in the high molecular weight fractions of G-75 from both photolyzed and nonphotolyzed samples. The large degree of covalent labeling in the dark was attributed to chemical reactions occurring during the long incubation period (72 h) and the high pH of the incubation solution. It was thus not possible to clearly distinguish between radiolabeling due to chemical reactions occurring in the dark or during photolysis. No labeling was observed in the control samples incubated with [^{125}I]glucagon.

Membrane Purification. The yield of partially purified membrane was 1–2 mg of protein/g wet weight of liver. The marker enzyme data demonstrated a 33-fold increase in the specific activity of 5′-nucleotidase in the partially purified membranes relative to the crude homogenate. The glucose 6-phosphatase and succinate–cytochrome *c* reductase activities indicate some microsomal and mitochondrial contamination, respectively, but no increase in their specific activities.

Adenylate Cyclase. Dose–response curves for the effect of native glucagon and of glucagon-NAPS on adenylate cyclase activity of hepatic plasma membranes are presented in Figure 5. The half-maximal stimulation occurs at 3.0×10^{-8} M for glucagon and at 2.5×10^{-8} M for glucagon-NAPS. NaF (20 mM) stimulated adenylate cyclase activity fourfold.

Discussion

The bifunctional reagent 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl) was synthesized by a series of five reactions which required inexpensive starting materials and which gave products in good yields. The structure of the final product was determined by proton NMR, IR, and mass spectra, elemental analysis, and spectrophotometric studies.

It has been reported previously that sulfenyl halides such as NPSCl and DNPSCl are highly specific reagents for tryptophan and cysteine residues of polypeptides and proteins under mildly acidic reaction conditions. The product of their reaction with tryptophan is a modified indole with a thioether function in position 2 of the ring (Scoffone et al., 1968;

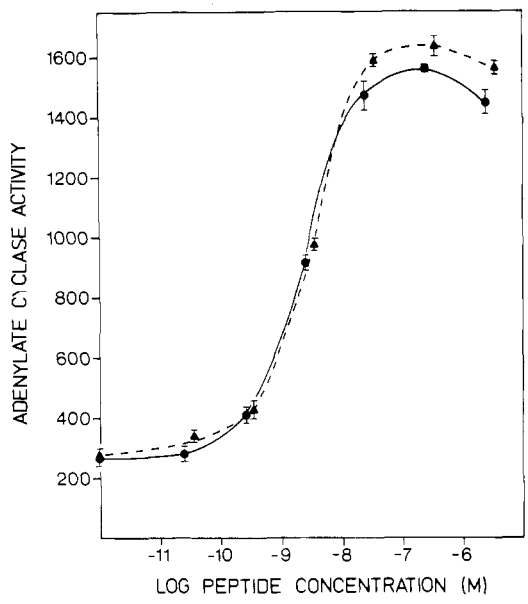


FIGURE 5: Effect of glucagon (▲) and glucagon-NAPS (●) concentrations on adenylate cyclase activity in partially purified plasma membrane. Assay conditions are described under Methods. Activity units are in pmol/(10 min·mg of protein). Vertical bars extend to the limits of the SEM for four determinations at each peptide concentration.

Fontana et al., 1968a,b; Veronese et al., 1968, 1970). The sulfenyl function is stabilized against hydrolysis by the presence of the nitro group in the ortho position of the aromatic ring, and sulfenamide formation is inhibited under acidic reaction conditions (Scoffone et al., 1968), thus ensuring efficiency and selectivity of the reaction. As expected, NAPSCl also reacts specifically with tryptophan in a mixture of amino acids not containing cysteine. The reaction with tryptophan is characterized by a second-order rate constant of $\sim 1.0 \times 10^2$ M^{-1} s^{-1}. There is also a small loss of methionine, however, which may be caused by oxidation as has been found in the case of NPSCl (Scoffone et al., 1968). In addition to tryptophan, sulfenyl halides can also convert cysteine into an unsymmetrical disulfide under similar reaction conditions. Cysteine can be reversibly recovered by treatment with a reducing agent (Fontana et al., 1968a,b). Even though the reaction of NAPSCl with cysteine was not investigated, it is believed that NAPSCl can effectively modify cysteine-containing proteins as well. Another potential bifunctional reagent for a selective modification of cysteine alone in polypeptides and proteins containing both cysteine and tryptophan could be in the intermeidate 2,2′-dinitro-4,4′-diazidodiphenyl disulfide. Reaction conditions for this reagent would require investigation because of the insolubility of the reagent in protic solvents.

Besides the specificity of NAPSCl for tryptophan-containing polypeptides and proteins that lack sulfhydryl groups, the greatest advantage is that it can be used in photolabeling studies of ligand–receptor interactions. This results from the presence of the azide moiety at the para position of the aromatic ring. As a nitrosulfenyl compound with absorption maxima in the visible region [NAPSCl, λ_{max} = 420 nm (ϵ = 2.95×10^3 M^{-1} cm^{-1})], a NAPS derivative can be photolyzed at long enough wavelengths so as to keep radiation damage to their targets minimal. The characteristic absorption spectra of NAPSCl and its derivatives before and after photolysis indicated that NAPS derivatives have the necessary properties for effective photolabels. The fact that aniline was not detected in the irradiation products of NAPSCl or 2,2′-dinitro-4,4′-diazidodiphenyl disulfide suggests that the major product of photoreaction is a singlet rather than a triplet state so that migration of the label through free-radical formation becomes less likely.

NAPSCl was shown to be specific and selective for the modification of the tryptophan residue in the hormone glucagon. Only one major species of glucagon modified at the single tryptophan-25 residue was obtained. The high yields of the product of the reaction of NAPSCl with glucagon indicated that NAPSCl can be an equally effective tryptophan-modifying reagent as NPSCl and DNPSCl (Scoffone et al., 1968; Epand & Cote, 1976). The characteristic absorption spectra of glucagon-NAPS (λ_{max} = 395 nm; ϵ = 3.6×10^3 M^{-1} cm^{-1}) and its destruction by photolysis indicated that it can be used for photoaffinity labeling studies.

The glucagon-NAPS analogue synthesized was compared to glucagon on a molar basis in its ability to stimulate liver adenylate cyclase and was found to be of a comparable activity. Similar results have been reported for the tryptophan derivatives, glucagon-NPS and $(glucagon)_2$DNPS (Epand & Cote, 1976), which indicate that an intact tryptophan residue is not essential for biological activity. The ability of glucagon-NAPS to activate adenylate cyclase makes the analogue an effective photoaffinity probe for the identification of the glucagon plasma membrane receptor. The photosensitive derivative of glucagon [^{125}I]-N^{ϵ}-(4-azido-2-nitrophenyl)glucagon (Bregman & Levy, 1977) has recently been used to covalently label the glucagon receptor. Even though the specific binding of that derivative to rat liver plasma membranes was comparable to that of native glucagon, it did not activate adenylate cyclase. It is believed that glucagon-NAPS has the advantage for receptor labeling in that the derivative is active so that binding sites for this derivative can be linked to physiological response. There is strong support that the hormone binding and an increased rate of cAMP formation are related processes requiring a receptor which can be coupled to the catalytic subunit of adenylate cyclase to form a functional complex in the plasma membrane (Lad et al., 1977; Welton et al., 1977; Schramm, 1979). Since the radioactively labeled glucagon-NAPS derivative was able to covalently label bovine serum albumin and anti-glucagon-IgG upon photolysis, this derivative can be effectively used as a photoaffinity probe in binding studies and in the identification of the glucagon receptor.

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