Use of a Nitrotryptophan-Containing Peptide for Photoaffinity Labeling the Pancreatic Cholecystokinin Receptor†

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ABSTRACT: We report the preparation and characterization of a new type of intrinsic photoaffinity labeling probe, on the basis of the incorporation of a photolabile nitrotryptophan into a biologically relevant domain of a peptide. The model system used was the pancreatic cholecystokinin (CCK) receptor, previously affinity labeled with a variety of probes. Those studies have suggested that an M_r = 85 000–95 000 protein is more likely to be labeled as the site of covalent attachment approaches the receptor-binding domain of this hormone. Indeed, CCK has a Trp in the center of its receptor-binding region, and replacement of that residue with 6-nitrotryptophan resulted in a photolabile probe which affinity labeled the same M_r = 85 000–95 000 pancreatic membrane protein. This probe, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33], was synthesized by solid-phase and solution techniques and characterized by mass spectrometry. Following oxidative iodination, it was purified on HPLC to 2000 Ci/mmol. Binding to pancreatic membranes was rapid, temperature dependent, reversible, saturable, and specific and was with high affinity (K_d = 3 nM). While its binding affinity was only 3-fold lower than that of native CCK-8, this probe was 70-fold less potent than native hormone in stimulating amylase secretion (EC_{50} = 1 nM) and equally efficacious to native hormone. Despite the slight decrease in affinity, this probe demonstrated a high relative efficiency of covalent labeling of the M_r = 85 000–95 000 protein. This confirms that the M_r = 85 000–95 000 protein represents the hormone-binding subunit of the CCK receptor and demonstrates the utility of this type of photoaffinity labeling probe. This should provide a generally applicable substitution for photoaffinity labeling of molecules in which a Trp resides in a domain relevant for the characterization of a bimolecular interaction.

Photoaffinity labeling is a powerful method for the biochemical characterization of molecules which interact with each other, such as ligands with their receptors or enzymes with their substrates. In this technique, after two molecules are allowed to interact, photolysis is used to activate a labile moiety in one so that it can generate a covalent bond to adjacent molecules. In this way, if the photolabile moiety can be introduced into the active site of one of these interacting molecules, it can be used to label that site on its target.

A number of methods have been used to introduce photolabile moieties into peptide ligands for photoaffinity labeling (Eberle & DeGraan, 1985). These include the acylation of ligands with nitrene and carbene precursors (Bayley, 1983); however, because of the bulky nature of these reagents, they must be introduced into nonrelevant regions of the native molecule. Of greater utility is the incorporation of modified amino acids directly into the peptide ligand (Escher et al., 1978; Mackiewicz et al., 1987; Powers et al., 1988a). It is important that such a substitution be as similar to the native residue as possible so as not to interfere with the nature of the bimolecular interaction. Recently, we have reported the substitution of *p*-nitrophenylalanine for Phe^{33} in a cholecystokinin (CCK) peptide for the photoaffinity labeling of the CCK receptor (Powers et al., 1988a). That probe identified the same M_r = 85 000–95 000 pancreatic plasma membrane protein as that previously labeled by decapeptide probes with sites of cross-linking at their amino terminus (Pearson & Miller, 1987; Pearson et al., 1987a), thus spanning the theoretical receptor-binding domain of this hormone (carboxyl-terminal heptapeptide) (Ondetti et al., 1970). This contrasts the labeling of a different candidate protein (M_r = 80 000) by use of a CCK-33-based probe with its site of cross-linking its amino terminus, a position far removed from the CCK receptor-binding domain (Pearson & Miller, 1987; Rosenzweig et al., 1983).

In this work, we have developed a new type of "intrinsic" photoaffinity labeling probe for the CCK receptor by modifying the Trp^{30} residue which is situated in the middle of the receptor-binding domain of this hormone. While a photolabile tryptophan derivative has been used for the labeling of *Escherichia coli* tryptophan synthetase (Miles & Phillips, 1985), no such derivative has previously been incorporated into a peptide ligand for photoaffinity labeling. Further, this should provide a generally applicable substitution for photoaffinity labeling of molecules in which a tryptophan resides in a domain relevant for the characterization of a bimolecular interaction.

Materials and Methods

Peptide Synthesis and Analytical Methods. Peptides were synthesized by a combination of solid-phase and solution techniques. Protected amino acids and methylbenzhydrylamine resin were from Chemical Dynamics (South Plainfield, NJ), Peninsula Laboratories (Belmont, CA), and Applied Biosystems (Foster City, CA).

Peptides were separated by reversed-phase high-performance liquid chromatography (HPLC). Semipreparative purifications were carried out on a 10-μm Vydac C-18 column (25 × 1 cm) with 300-Å pore size under a flow rate of 4 mL/min, while analytical separations were performed on a 5-μm Altex C-18 column (15 × 0.46 cm) under a flow rate of 1 mL/min. Both used triethylammonium acetate buffer (0.1 M), pH 5.0, a gradient being run from 10 to 60% acetonitrile over 50 min, with UV detection at 254 nm.

Melting points were obtained on a Mel-Temp apparatus and are uncorrected. Peptide hydrolyses were performed in 6 N

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Table I: Solid-Phase Synthesis Protocol

step	reagents and operation	mixing time (min)
1	DMF wash (five times)	1
2	20% piperidine in DMF	3 and 7
3	DMF wash (eight times)	1
4	Fmoc-protected amino acid (3 mmol), diisopropylcarbodiimide (3 mmol), and 1-hydroxybenzotriazole (3 mmol) in DMF (30 mL)	120
5	DMF wash (five times)	1
6	ninhydrin test	

HCl (Pierce Chemical Co., Rockford, IL) at 110 °C in a sealed vessel under high vacuum for 22 h. Analysis of the hydrolysates was performed on a Beckman 7300 amino acid analyzer (Palo Alto, CA).

6-NO_2-Trp (I). 6-NO_2-Trp was prepared by direct nitration according to the procedure of Moriya et al. (1975): mp 240 °C dec (literature mp 250–252 °C). Anal. Calcd for $C_{11}H_{11}N_3O_4$: C, 53.01%; H, 4.45%; N, 16.86%. Found: C, 52.87%; H, 4.41%; N 16.73%.

Fmoc-6-NO_2-Trp (II). 6-NO_2-Trp (5.0 g, 20 mmol) was suspended in 56 mL of 10% Na_2CO_3. Dioxane (60 mL) was added, and the suspension was cooled in an ice bath. Fmoc-Cl (5.18 g, 20 mmol) dissolved in 25 mL of dioxane was added dropwise to the suspension with vigorous stirring. The reaction was stirred for 2 h at 0–5 °C and then allowed to rise to room temperature. After standing overnight, the suspension was diluted with 400 mL of cold water, washed with ether, and acidified to pH 2 with concentrated HCl at 4 °C. The product was dried and triturated with ethyl acetate to give 5.95 g. Several crops were obtained by precipitation with hexanes from the ethyl acetate washes to give an additional 1.92 g. Overall yield was 7.87 g (84%); mp was 220 °C dec. Anal. Calcd for $C_{26}H_{20}N_3O_6$: C, 66.37%; H, 4.28%; N, 8.93%. Found: C, 65.37%; H, 4.92%; N, 8.81%.

Gly-Asp-Tyr(OSO_3)-Nle-Gly-(6-NO_2-Trp)-Nle-Asp-Phe-NH_2 (III). Methylbenzhydrylamine resin (4.44 g, 2 mequiv) was treated with pentafluorophenyl 4-(hydroxymethyl)-benzoate in 20 mL of dimethylformamide (DMF) for 1 h, washed with DMF and methanol, and dried in vacuo. The derivatized resin gave a negative Kaiser test (Kaiser et al., 1970). The (hydroxymethyl)phenyl groups were esterified with Fmoc-Phe-OH (4.64 g, 12 mmol), by use of diisopropylcarbodiimide (1.85 mL, 11.8 mmol), hydroxybenzotriazole (1.84 g, 12 mmol), and 4-(dimethylamino)pyridine (0.24 g, 2 mmol) in 20 mL of DMF for 2 h. Solid-phase peptide synthesis was carried out with Fmoc protection for α-amino groups and *tert*-butyl-based side-chain protection for Asp and Tyr, following the standard protocol given in Table I.

Side-chain protection was removed by suspending the resin in 95% TFA–2.5% thioanisole–2.5% ethanethiol (v/v) for 2 h at room temperature. Half of the resin (1 mequiv) was washed with chloroform, methanol, and pyridine and then treated with sulfur trioxide–pyridine complex (6.37 g, 40 mmol) in pyridine for 21 h. The resin was washed with pyridine, methanol, and DMF and the N-terminal Fmoc group was removed with 20% piperidine/DMF. The resin was washed with DMF and methanol and dried in vacuo.

This resin (1 mequiv) was suspended in a 50% anhydrous ammonia solution in dry methanol at −78 °C in a sealed vessel. The stirred suspension was allowed to rise to room temperature and ammonolysis carried out for 2 h. Solvents were removed by rotary evaporation, and the peptide was extracted with DMF, dried, dissolved in water, and lyophilized. Yield of crude peptide was 300 mg (25%).

The crude product contained approximately equal amounts of sulfated and unsulfated compound. The material was purified by semipreparative reversed-phase HPLC on octadecylsilica. Compound III eluted at 24.2 min from the analytical column under the system described above. Yield was 15 mg (5%).

D-Tyr-Gly-Asp-Tyr(OSO_3)-Nle-Gly-(6-NO_2-Trp)-Nle-Asp-Phe-NH_2 (IV). Nonapeptide III (12 mg, 9.9 mol) was acylated with *N,O*-bis(Fmoc)-D-Tyr-ONSu (12 mg, 17 mol) and deprotected as we have described (Powers et al., 1988b). The product was purified by HPLC (as described for compound III). Compound IV eluted at 24.9 min on the analytical column. Yield was 1.72 mg (12%).

Radioiodination. The peptide was iodinated with $Na^{125}I$ by use of the solid-phase oxidant *N*-chlorobenzenesulfonamide (Iodo-Beads, Pierce Chemical Co., Rockford, IL) (Pearson et al., 1986). A 5-μg aliquot was dissolved in 10 μL of methanol and 60 μL of 0.2 M sodium borate buffer, pH 9.0, containing $Na^{125}I$ (1 mCi), and one Iodo-Bead was added. After 30 s, 0.9 mL of 0.1 M triethylammonium acetate, pH 5.0, was added, and the reaction mixture was immediately aspirated from the reaction tube. This was loaded onto an octadecylsilane precolumn (Pierce), and the product was purified by reversed-phase HPLC using a Vydac C-18 column run isocratically at 1 mL/min with 30% acetonitrile–70% 0.1 M triethylammonium acetate, pH 5.0 (v/v).

Biological Activity. The biological activity of the unlabeled ligand, D-Tyr-Gly-[($Nle^{28,31}$,6-NO_2-Trp^{30})CCK-26–33], was determined by its ability to stimulate amylase secretion by dispersed pancreatic acini. The acini were prepared from 125–150-g male Harlan Sprague-Dawley rats by enzymatic and mechanical dissociation, according to the method of Schultz et al. (1980). Incubations were carried out in a modified Krebs–Ringers–Hepes (KRH) medium, pH 7.4, containing Hepes (25 mM), NaCl (104 mM), KCl (5 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2 mM), KH_2PO_4 (1 mM), D-glucose (2.5 mM), bovine serum albumin (0.2%), soybean trypsin inhibitor (0.01%), and essential and nonessential amino acids. One to five million acinar cells were incubated with various concentrations of secretagogue in 1 mL for 30 min at 37 °C. Media was separated from cells by centrifugation at 800*g*. Amylase concentration was determined by the generation of maltose from starch according to the method of Bernfeld (1951).

Pancreatic Membrane Preparation. Enriched pancreatic plasma membranes were prepared from similar animals according to the method we described (Rosenzweig et al., 1983). The excised pancreata were homogenized with a Dounce homogenizer at 4 °C in 10 volumes of 0.3 M sucrose containing 0.01% soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl fluoride, and 1 mM 2-mercaptoethanol. After filtration through gauze, the sucrose concentration of the homogenate was raised to 1.3 M by the addition of 2.0 M sucrose. This material was overlayered with 0.3 M sucrose and centrifuged for 3 h at 149000*g*. The membrane fraction at the 0.3 M–1.3 M sucrose interface was aspirated, washed twice, and stored in KRH medium at −70 °C. The protein concentration of the preparation was determined by fluorescamine assay using bovine serum albumin as standard (Udenfriend et al., 1972).

Binding Studies. Pancreatic plasma membranes containing 20 μg of protein and 18–22 pM radioligand were incubated with various concentrations of competing peptide for 60 min at 25 °C in 0.5 mL of KRH (conditions demonstrated to be at equilibrium). Bound radioligand was separated from free

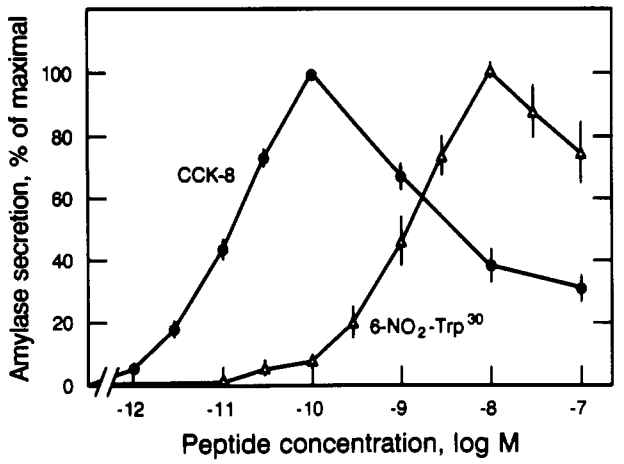

FIGURE 1: Both peptides, D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] and native CCK-8, stimulated dispersed pancreatic acini to secrete amylase with equal efficacy; however, D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] (EC_{50} = 1 nM) was less potent than CCK-8 (EC_{50} = 14 pM). Each point represents the mean ± SEM of four experiments performed in triplicate.

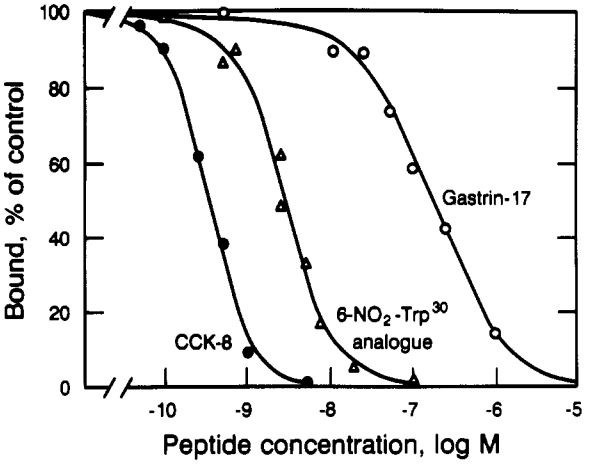

FIGURE 2: Structurally related peptides competed for binding of ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] to pancreatic plasma membranes in a concentration-dependent manner, while unrelated pancreatic ligands did not compete. Values represent the means of specific binding from three experiments performed in duplicate.

radioligand by rapid filtration using a Skatron cell harvester (Sterling, VA). Bound radioactivity was quantified on a γ spectrometer. Nonspecific binding was determined by simultaneous incubations in the presence of an excess (0.1 μM) of the unlabeled ligand.

Association experiments were performed in 3.6-mL incubations, removing duplicate 200-μL aliquots at specified times. The aliquots were analyzed by filtration as described above. Dissociation experiments were performed similarly, with the starting point being the suspension of membranes which had previously bound radioligand for 60 min at 25 °C into the appropriate temperature buffer which also contained 0.1 μM CCK-8.

In experiments designed to evaluate radioligand degradation, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] was incubated with pancreatic membranes for 60 min at 25 °C, followed by separation of bound from free radioligand. The bound radioligand was then allowed to dissociate into peptide-free media for 30 min at 37 °C. The supernatant from this incubation, containing radioligand which had dissociated from binding sites, was compared with fresh radioligand for ability to bind membranes.

Results

After synthesis, D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] was purified by HPLC to yield a sharp peak, and the structure was verified by amino acid analysis and mass spectrometry. As has been observed with other sulfated peptides (Arlandini et al., 1984), the most intense peak in the positive-ion ^{252}Cf plasma desorption mass spectrum corresponded to the desulfated ion. Calculated values (m/z) for $(M - SO_3 + Na)^+$ and $(M + 2Na - H)^+$ are 1315.36 and 1417.4, respectively, while values obtained were 1315.1 and 1417.9, respectively.

Biological Activity. D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})-CCK-26–33] stimulated dispersed rat pancreatic acini to secrete amylase with an efficacy similar to that of native CCK-8, but with lower potency (70-fold less) (Figure 1). The concentration–response curves for native CCK-8 and for this compound were shaped similarly, with both exhibiting the typical increase to maximal secretion, with inhibition of secretion by supramaximal concentrations of peptide.

Binding Characterization. ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] bound to rat pancreatic membranes in a high-affinity, saturable, specific manner, with nonspecific binding in the presence of 0.1 μM unlabeled CCK-8 representing less than 20% of total binding. Structurally related analogues, CCK-8, unlabeled D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33], and gastrin 17, inhibited this binding in a concentration-dependent manner (Figure 2). Unrelated pancreatic secretagogues (VIP, secretin) did not modify binding, even when incubated in a concentration as high as 1 μM.

Binding association and dissociation were temperature dependent (Figure 3). Association was initially more rapid at 37 °C than at 25 °C and more rapid at 25 °C than at 4 °C. However, binding at 37 °C decreased after a prolonged incubation period, whereas that at 25 °C reached a plateau after 45 min. Dissociation was much faster at 25 °C than at 4 °C.

To assess the stability of the radioligand in binding studies, we compared the abilities of dissociated ligand and fresh ligand to bind to pancreatic membranes. ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] fully maintained its ability to bind after previous interaction with its receptor (102 ± 5%).

Photoaffinity Labeling. In order to assess optimal photolysis conditions for affinity labeling, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] was bound to pancreatic membranes to equilibrium and then exposed to ultraviolet light for different times, and the membrane proteins were subsequently resolved on SDS–polyacrylamide gels and visualized by autoradiography (Figure 4a). Covalent attachment of this ligand to a M_r = 85 000–95 000 protein was apparent after only 5 min of photolysis, with incorporation at 10 min representing 63% of the maximum reached after 45 min. Depending on the photolysis time between 5 and 45 min, 1.27%–3.5% of the specifically bound radioactivity was recovered in the labeled band (Figure 4b). The efficiency of incorporation decreased after prolonged UV irradiation of 60 and 90 min. Labeling was dependent on photolysis, with no covalent attachment observed when light was absent or when the ligand was photolyzed before being added to the incubation. In addition, the covalent attachment was due to the nitrotryptophan moiety, since the analogous probe with unmodified tryptophan generated little labeling under similar photolytic conditions (Figure 4).

CCK-8 competed for the labeling of the M_r = 85 000–95 000 protein in a concentration-dependent manner, with 50% inhibition in the presence of 1 nM CCK-8 (Figure 5). The

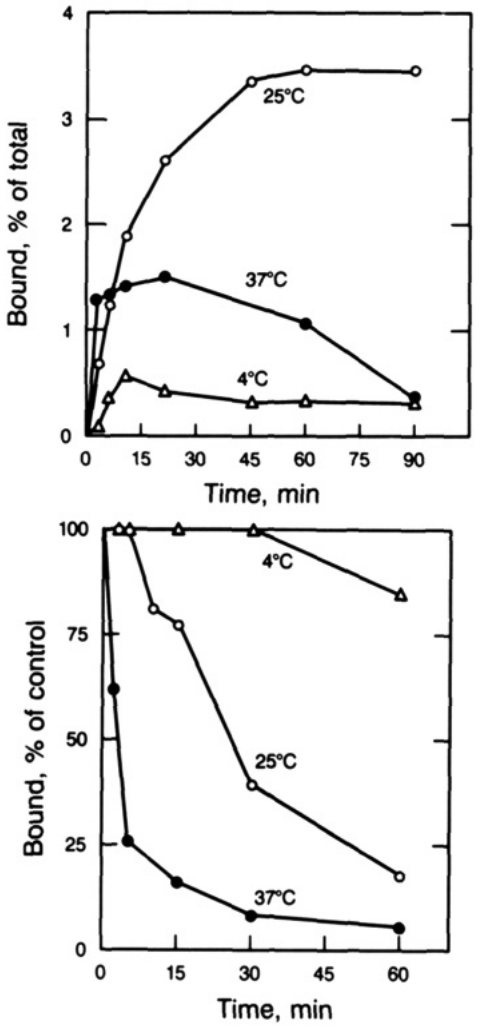

FIGURE 3: Time and temperature dependence of association and dissociation of ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] binding to pancreatic plasma membranes. In the association experiments, (top), specific binding of radioligand is plotted as a percentage of total radioligand in the incubation. In dissociation experiments (bottom), radioligand remaining bound is expressed as a percentage of that specifically bound at the beginning of the experiment. Values represent the means of three experiments performed in duplicate.

electrophoretic migration of this band was not different when the membranes had been prepared in the strict absence or presence of reducing agents or when 100 mM dithiothreitol was added to the sample buffer, suggesting that the labeled protein was not composed of subunits or part of a larger complex linked with disulfide bonds.

Endo F deglycosylation of the M_r = 85 000–95 000 band labeled by ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] shifted its migration to M_r = 42 000 (Figure 6), thus confirming the identity of the M_r = 85 000–95 000 species as the N-linked glycoprotein previously labeled with other CCK receptor probes (Powers et al., 1988a; Pearson & Miller, 1987). This band comigrated on SDS–polyacrylamide gels with the deglycosylation products we described previously using CCK decapeptide probes cross-linked through their amino terminus (Pearson & Miller, 1987) or carboxyl terminus (Powers et al., 1988a).

Discussion

In this work, we have been successful in modifying a tryptophan residue in the middle of the receptor-binding domain of the peptide hormone cholecystokinin (CCK) to generate a photolabile probe for the CCK receptor. Replacing Trp^{30} in a CCK analogue with a 6-nitrotryptophan residue was a well-tolerated substitution, maintaining full biological efficacy and good binding affinity. The 6-nitrotryptophan residue was adequately photolabile to form covalent attachment to adjacent molecules, while being stable enough to be handled easily under laboratory conditions.

The receptor-binding domain of CCK has been well localized to the carboxyl-terminal heptapeptide on the basis of the extensive primary structure–activity information (Ondetti et al., 1970; Villanueva et al., 1982). The amino terminus of this domain can be freely modified, while maintaining full binding and biological activities (Ondetti et al., 1970; Miller et al., 1981). This suggests that the amino terminus of CCK-33 (the site of covalent attachment when affinity labeling with a CCK-33-based probe) may be far removed from the CCK receptor. While such a probe was the first to be used for the biochemical characterization of the pancreatic CCK receptor (Rosenzweig et al., 1982, 1983), it labeled a plasma membrane glycoprotein of approximate M_r = 80 000 (variably described to be between M_r = 76 000 and M_r = 95 000)

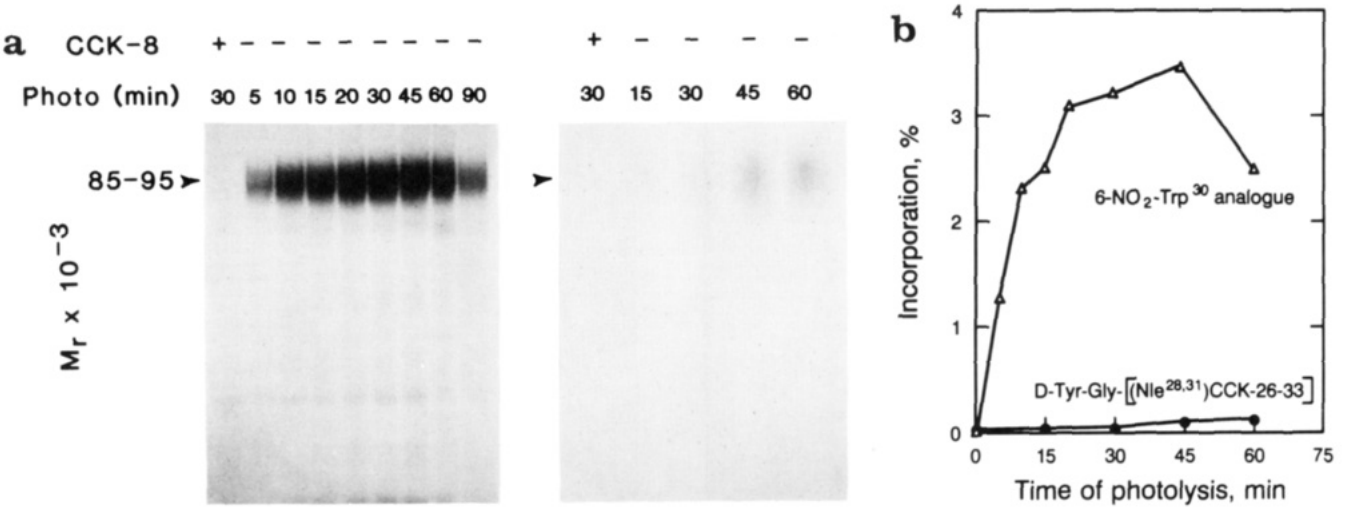

FIGURE 4: (a) Similar amounts of ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] (left) and ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26–33] (right) were bound to pancreatic plasma membranes to equilibrium and photolyzed for the specified times. The membranes were then solubilized, resolved on SDS gels, and visualized by autoradiography. The times of exposure were 48 h (left) and 14 days (right), suggesting the much greater photolytic yield with the nitrotryptophan-containing peptide. This was quantified in (b) where the M_r = 85 000–95 000 labeled bands were cut from the gels and counted. Values represent the percentages of specifically bound counts that were present in the labeled bands.

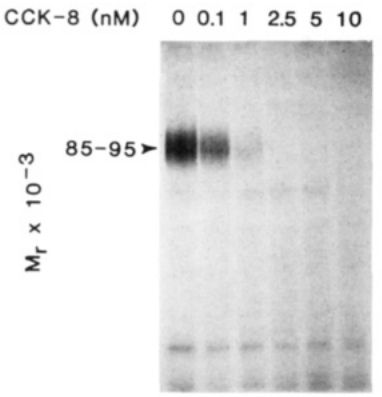

FIGURE 5: CCK-8 competed for the labeling of the M_r = 85000–95000 pancreatic membrane protein by ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] in a concentration-dependent manner, with more than 50% of labeling inhibited by 1 nM CCK-8.

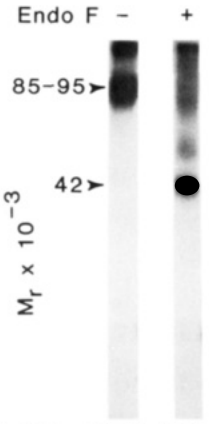

FIGURE 6: Affinity labeled M_r = 85000–95000 pancreatic membrane protein was cut from a gel and electroeluted prior to deglycosylation with endoglycosidase F. The electroeluted band migrated in the same position on the second gel (lane 1), while Endo F treatment shifted it to M_r = 42000 (lane 2), the same position as the product of Endo F treatment of the previously labeled receptor candidate protein (Powers et al., 1988a; Pearson & Miller, 1987).

(Rosenzweig et al., 1982, 1983, 1984; Madison et al., 1984; Sakamoto et al., 1983). Subsequently, we developed a series of "shorter" decapeptide probes which could be cross-linked through their amino-terminal regions, which were much closer to the receptor-binding domain (Pearson & Miller, 1987; Pearson et al., 1987a). These probes labeled a distinct glycoprotein of M_r = 85000–95000, which had a different size protein core from the previously labeled protein (Pearson et al., 1987b). Further supporting the identification of the M_r = 85000–95000 protein as the hormone-binding subunit of the pancreatic CCK receptor were our studies using a carboxyl-terminal photoaffinity labeling probe to also identify this protein (Powers et al., 1988a).

Despite suggestions that the Trp^{30} residue is important for intramolecular interactions to confer stable structure to CCK (Adachi et al., 1981), previous structure–activity studies of the Trp^{30} residue of CCK suggest that certain modifications can be introduced into this residue in the middle of the receptor-binding domain without dramatic loss of binding or biological activities (Adachi et al., 1981; Penke et al., 1984; Rajh et al., 1980). Replacement of the hydrogens in position 5 or 6 of the indole ring of Trp^{30} with fluorine does not alter the potency of these CCK analogues to stimulate pancreatic enzyme secretion (Rajh et al., 1980). Also, methylation of the Trp^{30} indole nitrogen has been reported to lower potency of this analogue only by a factor of 30 (Adachi et al., 1981).

The photolability of nitro and azido derivatives of phenyl rings are well established (Bayley, 1983). The nitro derivatives are less photolabile and more chemically stable than the azido derivatives (Bayley, 1983). Our ability to previously perform photoaffinity labeling of the pancreatic and gallbladder CCK receptors using a *p*-nitrophenylanine-33 derivative of CCK (Powers et al., 1988a; Schjoldager et al., 1988) suggested that a nitrotryptophan derivative of this hormone would be adequately labile for those studies. Indeed, the relative stability of this analogue could be an advantage for ease of use of the compound.

Indeed, D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] was found to bind to the pancreatic CCK receptor in a rapid, temperature-dependent, saturable, specific, high-affinity manner. The observation that this peptide possessed better binding activity than biological potency was not too surprising. We might anticipate that the receptor-binding domain of a ligand would be particularly sensitive to any structural modifications. This analogue had enough features of native CCK to be recognized to bind but not enough to be very potent at activating the stimulus-secretion cascade.

We would anticipate that the 6-azidotryptophan-30 derivative of this peptide would also be a tolerated substitution, useful for photoaffinity labeling studies. However, that derivative is much more photolabile and much more difficult to handle in the general laboratory. In preliminary studies with an azido derivative, we observed labeling of the same pancreatic membrane protein as that reported in this paper and recognized no advantages to its use.

An additional advantage of the siting of the photolabile moiety within the theoretical receptor-binding domain was the relatively high efficiency of covalent labeling of the receptor. Though it had a lower affinity for binding than other probes with sites of photolysis outside of this domain (Pearson & Miller, 1987; Pearson et al., 1987a), this probe was more efficient at labeling the CCK receptor.

The current observation of the labeling of the pancreatic plasma membrane M_r = 85000–95000 protein with ^{125}I-D-Tyr-Gly-[(Nle^{28,31},6-NO_2-Trp^{30})CCK-26–33] provides important complementary data confirming that this represents the hormone-binding subunit of the CCK receptor. With this observation, that glycoprotein has been specifically labeled by photoaffinity labeling probes with sites of covalent attachment at the amino terminus (Pearson et al., 1987a), carboxyl terminus (Powers et al., 1988a), and midregion of the receptor-binding domain of CCK. Photoaffinity labeling techniques have been successfully used to characterize the domains which are important for molecular interactions (Kerlavage & Taylor, 1980; Lifter et al., 1974; Wong et al., 1988). As the sequence of the CCK receptor becomes available, "intrinsic" photoaffinity labeling probes, such as the one we currently report, may provide similar molecular detail about this receptor.

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REFERENCES

Adachi, H., Rajh, H. M., Tesser, G. I., DePont, J. J. H. H. M., Jensen, R. T., & Gardner, J. D. (1981) *Biochim. Biophys. Acta 678*, 358–363.

Arlandini, E., Gioia, B., Perseo, G., & Vigevani, A. (1984) *Int. J. Pept. Protein Res. 24*, 386–391.

Bayley, H. (1983) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., & Burdon, R. H., Eds.) Vol. 12, pp 8–14, Elsevier, Amsterdam and New York.

Bernfeld, P. (1951) *Adv. Enzymol. Relat. Areas Mol. Biol. 12*, 379–428.

Eberle, A. N., & DeGraan, P. N. E. (1985) *Methods Enzymol. 109*, 129–156.

Elder, J. H., & Alexander, S., (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4540–4544.

Escher, E., Nguyen, T. M. D., Robert, H., St.-Pierre, S. A., & Regoli, D. (1978) *J. Med. Chem. 21*, 860–864.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 117*, 595–598.

Kerlavage, A. R., & Taylor, S. S., (1980) *J. Biol. Chem. 255*, 8483–8488.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lifter, J., Hew, C.-L., Yoshioka, M., Richards, F. F., & Konigsberg, W. H. (1974) *Biochemistry 13*, 3567–3571.

Mackiewicz, A., Belisle, S., Bellabarba, D., Gallo-Payet, N., Lehoux, J.-G., Lagace, G., & Escher, E. (1987) *Helv. Chim. Acta 70*, 423–429.

Madison, L. D., Rosenzweig, S. A., & Jamieson, J. D. (1984) *J. Biol. Chem. 259*, 14818–14823.

Miles, E. W., & Phillips, R. S. (1985) *Biochemistry 24*, 4694–4703.

Miller, L. J., Rosenzweig, S. A., & Jamieson, J. D. (1981) *J. Biol. Chem. 256*, 12417–12423.

Moriya, T., Hagio, K., & Yoneda, N. (1975) *Bull. Chem. Soc. Jpn. 48*, 2217–2218.

Ondetti, M. A., Rubin, B., Engel, S. L., Pluscec, J., & Sheehan, J. T. (1970) *Am. J. Dig. Dis. 15*, 149–156.

Pearson, R. K., & Miller, L. J. (1987) *J. Biol. Chem. 262*, 869–876.

Pearson, R. K., Hadac, E. M., & Miller, L. J. (1986) *Gastroenterology 90*, 1985–1991.

Pearson, R. K., Miller, L. J., Powers, S. P., & Hadac, E. M. (1987a) *Pancreas 2*, 79–84.

Pearson, R. K., Miller, L. J., Hadac, E. M., & Powers, S. P. (1987b) *J. Biol. Chem. 262*, 13850–13856.

Penke, B., Hajnal, F., Lonovics, J., Holzinger, G., Kadar, T., Telegdy, G., & Rivier, J. (1984) *J. Med. Chem. 27*, 845–849.

Powers, S. P., Fourmy, D., Gaisano, H., & Miller, L. J. (1988a) *J. Biol. Chem. 263*, 5295–5300.

Powers, S. P., Pinon, D. I., & Miller, L. J. (1988b) *Int. J. Pept. Protein Res. 31*, 429–434.

Rajh, H. M., Smyth, M. J., Renckens, B. A. M., Jansen, J. W. C. M., DePont, J. J. H. H., Bonting, S. L., Tesser, G. I., & Nivard, R. J. F. (1980) *Biochim. Biophys. Acta 632*, 386–398.

Rosenzweig, S. A., Miller, L. J., & Jamieson, J. D. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1183.

Rosenzweig, S. A., Miller, L. J., & Jamieson, J. D. (1983) *J. Cell Biol. 96*, 1288–1297.

Rosenzweig, S. A., Madison, L. D., & Jamieson, J. D. (1984) *J. Cell Biol. 99*, 1110–1116.

Sakamoto, C., Goldfine, I. D., & Williams, J. A. (1983) *J. Biol. Chem. 258*, 12707–12711.

Schjoldager, B., Powers, S. P., & Miller, L. J. (1988) *Am. J. Physiol. 255*, G579–G586.

Schultz, G. S., Sarras, M. P., Gunther, G. R., Hull, B. E., Alicea, H. A., Gorelick, F. S., & Jamieson, J. D. (1980) *Exp. Cell Res. 130*, 49–62.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Limgruber, W., & Weigele, M. (1972) *Science 178*, 871–872.

Villanueva, M. L., Collins, S. M., Jensen, R. T., & Gardner, J. D. (1982) *Am. J. Physiol. 242*, G416–G422.

Wong, S. K.-F., Slaughter, C., Ruoho, A. E., & Ross, E. M. (1988) *J. Biol. Chem. 263*, 7925–7928.