Identification of the guanine binding domain peptide of the GTP-binding site of glucagon

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Abstract

Glucagon, a peptide hormone synthesized and secreted by α islet cells, regulates glucose homeostasis by several mechanisms. Using [γ - 32 P]8N₃GTP, a proven photoaffinity probe for GTP, a specific nucleotide binding site on human glucagon was detected that showed preference for GTP. Half-maximal saturation of photoinsertion into the polypeptide hormone was at 8–12 μ M with either [α - 32 P]8N₃GTP or [γ - 32 P]8N₃GTP. GTP protected photolabeling with an apparent k_d of 15 μ M, whereas ATP was less effective as a protector, exhibiting an apparent k_d of about 30 μ M. Maximal protection by GTP and ATP was over 90%. UTP, CTP, GDP, ADP, GMP, AMP, guanosine, adenosine, guanine, and adenine were much less effective protectors, indicating that binding is specific for purine nucleoside triphosphates, particularly GTP. Mg²⁺ at 150 μ M enhanced photoinsertion (twofold), whereas at 2–10 mM, it inhibited photoinsertion. Both Ca²⁺ and Zn²⁺ at 0.2 mM decreased photoinsertion about 45%. Purification of chymotryptic and tryptic digests of photolabeled glucagon by reverse-phase high performance liquid chromatography (HPLC) revealed that the N-terminal peptide, HSQGTF, was the only peptide region covalently photomodified by [32 P]8N₃GTP. GTP, if present during photolysis, greatly reduced both photoinsertion into glucagon and the amount of radiolabeled peptide recovered on HPLC. The specificity of binding to the N-terminal region is suggestive of a physiological role for a glucagon–GTP complex in the mechanism of action of this hormone.

Keywords: active-site peptide; glucagon; GTP; photoaffinity

Glucagon, a 3.4-kDa peptide hormone secreted by the α cells of the islet of Langerhans, modulates glucose homeostasis through interactions with receptors on hepatic cells. The nature of association of glucagon with the hepatic glucagon receptors is complex because both low- and high-affinity binding sites have been described (Mason & Tager, 1985). It is known that under appropriate conditions glucagon has the ability to stimulate hepatic cell adenylate cyclase by a mechanism that is modulated by GTP binding to a G-regulatory protein at micromolar concentrations (Rodbell et al., 1971). Termination of this hormone-mediated signal is less well defined but is thought to involve the hydrolysis of GTP, resulting in an inactive GDP–G-regulatory protein complex. Recent studies have also implicated glucagon-specific proteases at the receptor level on the plasma membrane as acting

on the hormone (Sheetz & Tager, 1988a,b). Some studies have shown processing of glucagon to be inhibited by GTP with a half-maximal inhibition at 6.5 μ M (Sheetz & Tager, 1988a). Whether or not this processing is the direct cause of “turning off” the glucagon-mediated signal is unclear. Another possible mechanism of processing is receptor-mediated endocytosis and subsequent degradation (Barazzzone et al., 1980; Watanabe et al., 1984). A specific role for GTP in mediating the glucagon–receptor interaction and glucagon metabolism appears to be well established. However, the mechanism of GTP involvement remains unresolved.

Korn and Ottensmeyer have predicted through Corey–Pauling–Koltun space-filling models that glucagon contains a cleft in three-dimensional analyses that could support GTP binding (Korn & Ottensmeyer, 1983). Also, in dialysis experiments, glucagon was able to concentrate [3 H]GTPpCH₂p, a radioactive nonhydrolyzable analog of GTP (Korn & Ottensmeyer, 1983). Other studies have suggested some structural rigidity in glucagon but indi-

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cate that the hormone may adopt different conformations under different conditions (Boesch et al., 1978; Braun et al., 1981).

Earlier work with $8N_3$ GTP has shown that this photoaffinity analog of GTP can, in the absence of activating light, substitute for GTP in many biochemical systems including tubulin polymerization (Geahlen & Haley, 1979) and activation of adenylate cyclase (Hoyer et al., 1986). In the latter report a potent GTP analog inhibitor of adenylate cyclase effectively displaced the activating $8N_3$ GTP from a 43,000 M_r protein at concentrations correlating with the induced inhibition. The selectivity of this probe was also demonstrated using human control and Alzheimer's-diseased brain homogenates. Within these complex homogenates only GTP binding proteins were photolabeled with $[^{32}P]8N_3$ GTP, and specific differences between Alzheimer's and control brains could easily be detected (Khatoon et al., 1989). These reports indicate that $8N_3$ GTP could be effectively used to study GTP-regulated events such as those involving glucagon. Recent studies with other biological response modifiers, such as atrial natriuretic factor (Mann et al., 1991), and interleukin-2 (IL-2) (Campbell et al., 1990), have shown that they contain specific nucleotide binding sites. It has been speculated that many biological response modifiers, including glucagon, are internalized and exposed to the intracellular milieu (Barazzzone et al., 1980; Watanabe et al., 1984). It is therefore possible that the nucleotide binding properties of these molecules may modulate some aspects of their biological activity. These aspects may be nucleotide-enhanced dissociation of the polypeptide from the internalized receptor or perpetuation of the signal by some nucleotide-mediated regulatory or catalytic event. Other effects are also possible, such as a decreased rate of processing of the bound biological response modifier.

In the present study, using the photoaffinity probe $[^{32}P]8N_3$ GTP, glucagon is shown to contain a specific GTP binding site of high affinity, which is sensitive to pH changes and divalent metal ion concentration. Both chymotryptic and tryptic digestion of glucagon photolabeled with $[^{32}P]8N_3$ GTP, followed by reverse-phase high performance liquid chromatographic (HPLC) purification, produced a single photolabeled peptide each. These peptides contained an overlapping sequence, and the guanine base binding domain was determined to involve at least partially the six amino acid residues in the N-terminal fragment of glucagon.

Results

The specificity of nucleotide interaction with glucagon can be demonstrated by saturation and protection studies. Both $[^{32}P]8N_3$ GTP and $[\alpha\text{-}^{32}P]8N_3$ GTP maximally photoinserted into glucagon at 25–30 μ M with an apparent k_d between 8 and 12 μ M (Fig. 1). When the sample was not irradiated, no labeling was observed, even for in-

Fig. 1. Saturation of $[^{32}P]8N_3$ GTP and $[^{32}P]8N_3$ ATP photoinsertion into glucagon. One microgram of glucagon in 60 μ L of reaction buffer was photolyzed with the indicated concentration of azidonucleotide and subjected to SDS-PAGE. ^{32}P incorporation was detected by autoradiography and quantified by cutting out the appropriate band and determining radioactivity by liquid scintillation counting. One hundred percent photoinsertion corresponds to 1.3×10^4 and 1.05×10^4 cpm for $[^{32}P]8N_3$ GTP and $[^{32}P]8N_3$ ATP, respectively. Closed circles, $[^{32}P]8N_3$ GTP; open circles, $[^{32}P]8N_3$ ATP.

cubation times up to 5 min. This indicates the absence of phosphorylation under these conditions. Prephotolysis of the probe, followed by immediate addition of the hormone, produced no covalent modification. This eliminates any pseudophotoaffinity labeling by light-generated long-lived chemical intermediates. The observed k_d value for the glucagon–GTP interaction is near the reported levels of GTP (6.5 μ M) that half-maximally inhibit glucagon from both interacting with its receptor as well as being processed by an endogenous membrane protease (Sheetz & Tager, 1988a).

Photolabeling was determined to be within a GTP-specific site by the following. Photolabeling glucagon with 25 μ M $[^{32}P]8N_3$ GTP was inhibited by GTP with an apparent k_d of 15 μ M. About 90% protection was achieved at 210 μ M GTP. This is the most theoretically obtainable, as both GTP and $8N_3$ GTP bind with about the same affinity, and about 12% of the total nucleotide is photoprobe. As shown in Figure 2, protection of photolabeling with $[^{32}P]8N_3$ GTP was greatest with GTP or $8N_3$ GTP. Other nucleotides that were not as effective protected in the following order: ATP > GDP > GMP > guanosine > guanine. $8N_3$ GTP bound with an affinity almost identical to GTP. This ability of $8N_3$ GTP to mimic GTP has been observed with other GTP-modulated systems.

Saturation of photoinsertion of $[^{32}P]8N_3$ ATP into glucagon occurred at about 80 μ M concentration with

Fig. 2. Prevention of $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ photoinsertion into glucagon by guanine nucleotides. Twenty micromolar $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ was incubated with $1\ \mu\text{g}$ of glucagon in the presence of the indicated concentration of various guanine nucleotide derivatives, and the reaction was photolyzed. ^{32}P incorporation was determined as described in Figure 1. **Inset:** Autoradiogram of a gel in which GTP was the competitor. Lanes 1–8: 0, 5, 10, 25, 40, 75, 100, and $210\ \mu\text{M}$ GTP, respectively. For clarity, data points corresponding to $5\ \mu\text{M}$ competitor were not plotted.

an apparent k_d of $28\ \mu\text{M}$ (Fig. 1). Photoinsertion of $[\gamma^{32}\text{P}]\text{8N}_3\text{ATP}$ into glucagon was reduced by preincubation of the hormone with ATP (Table 1). However, GTP and $8\text{N}_3\text{GTP}$ protected better than ATP. This indicates that $[\gamma^{32}\text{P}]\text{8N}_3\text{ATP}$ is probably interacting at the GTP site. ADP and AMP did not protect nearly as well. Adenosine and $8\text{N}_3\text{adenosine}$ were poor protectors, al-

Table 1. Effect of competitors ($200\ \mu\text{M}$) on photolabeling of glucagon with $40\ \mu\text{M}$ $[\gamma^{32}\text{P}]\text{8N}_3\text{ATP}$ ^a

	% Control
None	100
Adenine	99
Adenosine	100
$8\text{N}_3\text{Adenosine}$	97
$5'\text{AMP}$	82
ADP	65
ATP	33
$8\text{N}_3\text{ATP}$	22
GTP	21
$8\text{N}_3\text{GTP}$	25

^a These experiments were repeated at least three times each. Each number represents an average of three values and is accurate within $\pm 10\%$.

though $8\text{N}_3\text{adenosine}$ was slightly better than adenosine at very high concentrations ($600\ \mu\text{M}$, data not shown). It also appears as if $8\text{N}_3\text{ATP}$ binds more tightly than ATP, which suggests slightly enhanced binding due to the presence of the azide on the adenine ring. The pyrimidine nucleotides, UTP and CTP, had no effect on photolabeling with either $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ or $[\gamma^{32}\text{P}]\text{8N}_3\text{ATP}$ (data not shown), suggesting that the site is specific for purine nucleoside triphosphates. Also, lack of protection by GMP, AMP, CTP, or UTP eliminates screening of UV light as a cause for decreased photoinsertion by GTP.

Photoinsertion with $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ was optimal at pH 5.0–5.5 in MES buffer (data not shown). Photoinsertion was reduced nearly 50% at neutral pH, and no significant photoinsertion occurred at pH values above 7.5. These results were also obtained in a $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer system over a range of pH from 3.0 to 8.0 (data not shown). Mg^{2+} at low ($250\ \mu\text{M}$) concentrations caused an increase in $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ photoinsertion into glucagon of almost twofold. Consistent with this effect, $5\ \text{mM}$ EDTA reduced photoinsertion by 50%. However, Mg^{2+} at $10\ \text{mM}$ reduced photoinsertion over 90%, and Ca^{2+} or Zn^{2+} at $5\ \text{mM}$ reduced photoinsertion about 50%, whereas K^+ in the range of 1–5 mM had no effect (Table 2).

To determine the extent of multiple photolyses on glucagon photolabeling, $100\ \mu\text{g}$ ($29\ \text{nmol}$) of glucagon were incubated with $40\ \mu\text{M}$ $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ and photolyzed. This step was repeated two more times. With each stepwise photolabeling, about a 20% increase of photolabeling was observed as determined by laser densitometric analysis of autoradiograms made from sodium dodecyl sulfate (SDS) gels on which glucagon had been separated from reaction media. Prior to these experiments, it was shown that preirradiation of glucagon, before the addition of photoprobe, had no effect on the extent of photoinsertion when photoprobe was subsequently added and photolyzed.

Glucagon modified with $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ had a slightly lower retention time on reverse-phase HPLC compared

Table 2. Effect of metal ions and chelators on photolabeling of glucagon with $40\ \mu\text{M}$ $[\gamma^{32}\text{P}]\text{8N}_3\text{GTP}$ ^a

	Concentration (mM)			
	0.25	2.5	5.0	7.5
EDTA	N.D. ^b	95	76	61
MgCl_2	190	47	33	27
CaCl_2	N.D.	79	51	31
KCl	N.D.	98	95	N.D.

^a These experiments were repeated at least three times each. Each number represents an average of three values and is accurate within 10%.

^b N.D., not determined

to nonphotolabeled glucagon. It was determined that at least 30–40% of the glucagon recovered by HPLC was modified with [γ - 32 P]8N₃GTP after three photolyses (data not shown). However, this is likely an underestimation due to the lability of the covalently attached probe during reverse-phase HPLC analysis as seen in Figure 4 (King et al., 1991).

To determine the sequence within glucagon that was modified by photoinsertion with [γ - 32 P]8N₃GTP, glucagon was photolyzed three times as described above. However, radioactive 8N₃GTP was used only in the first photolysis, and nonradioactive 8N₃GTP was used in the two subsequent photolyses. This would decrease any non-specific radiolabeling while increasing the amount of specific photolabeled peptide. This procedure identified the

radiolabeled peptide with about 10^6 cpm of 32 P photoinserted, which was more than adequate for detection.

Complete proteolysis of glucagon by trypsin should produce three peptides as should proteolysis by chymotrypsin. Digestion of photolabeled glucagon was complete within 4 h as determined by both analysis of autoradiograms, made from SDS-polyacrylamide gel electrophoresis (PAGE) on which glucagon peptides were separated, and analysis by reverse-phase HPLC.

Multiply photolabeled glucagon was treated with trypsin and subjected to reverse-phase HPLC as described in the Materials and methods. There were five UV-absorbing peaks produced on HPLC (Fig. 3A). Two of these peaks are due to incomplete digestion, and the other three represent predicted peptide fragments. Only one of the

Fig. 3. HPLC analysis of tryptic fragments from glucagon modified by [γ - 32 P]8N₃GTP. **A:** UV absorbance (210 nm) profile of tryptic digests from photolabeled glucagon on reverse-phase HPLC. **B:** Radioactive profile of photolabeled glucagon from HPLC. **C:** Radioactive profile of photolabeled glucagon preincubated with 100 μ M GTP.

tryptic peptide peaks contained significant radiolabel, which was about 33% of the total cpm. Over 60% of the cpm eluted in the flow through. However, no peptide was detected in this region. The flow-through radioactive peak represents unbound photolyzed [γ - 32 P]8N₃GTP and some loss of covalently inserted [γ - 32 P]8N₃GTP from the photolabeled glucagon fragments during HPLC. The radiolabeled peptide eluted at 32 min, which corresponds to 36% buffer B (Fig. 3). This peak was sequenced and found to be the N-terminal tryptic fragment of glucagon (residues 1–12, HSQGTFTSDYSK). When glucagon was preincubated with 100 μ M GTP prior to photolysis, the UV-absorbing peptide peak remained, but there was no radioactivity associated with it (Fig. 3).

Fragmentation of photolabeled glucagon by chymotrypsin was used to narrow the site of photoinsertion, as the photomodified tryptic peptide contained an aromatic residue at position 6. This should also change the elution time of the peptide, and the radiolabel should comigrate with the new peak. Digestion by chymotrypsin should also eliminate the possibility of any radioactive artifact that just happened to comigrate with the tryptic fragment. Chymotrypsin treatment of modified glucagon produced four UV-absorbing fragments on HPLC. As with trypsin digestion, only one radioactive peptide peak was obtained that contained 40% of the cpm compared to that of the flow through. Because there were other UV-absorbing peaks in the vicinity, this radioactive peak was evaporated to remove acetonitrile and subjected to another HPLC run with a shallower gradient as described in the Materials and methods. One peptide peak was obtained, which eluted at 26 min, corresponding to 27% solvent B. Radioactivity coeluted with this peak (Fig. 4). This peak was sequenced and it corresponded to residues 1–6 (HSQGTF) of the N-terminal region of glucagon (see Table 3). Each of the experiments reported above were repeated 2–3 times with confirming results. Due to the problem of radiolabel not being stable to HPLC (see Fig. 4 where isolated peptide was rechromatographed and some radiolabel came out in the void volume) or to sequence analysis methods (King et al., 1991), the modified amino acid residue(s) could not be absolutely identified.

Discussion

Glucagon, a regulator of glucose homeostasis, acts through its receptor to activate adenylate cyclase. The subsequent processing of the hormone and halting of the glucagon-mediated signal is not yet fully understood. The three-dimensional structure of glucagon has been predicted to contain a GTP binding site in which the hydrophobic residues Tyr-10 and Phe-22 form a hydrophobic groove whereby the guanine ring of GTP is able to bind (Korn & Ottensmeyer, 1983). Our results suggest that the guanosine binding domain of the GTP binding site is located within proximity of the N-terminus. However, hy-

Fig. 4. HPLC analysis of chymotryptic fragments from glucagon modified by [γ - 32 P]8N₃GTP. UV absorbance (210 nm) and radioactive profiles of the radiolabeled peak from a previous HPLC run that contained chymotryptic digests of photolabeled glucagon. The gradient on the second run was shallower (see text and Materials and methods).

drophobicity introduced by residues Tyr-10 and Phe-22 may contribute to binding, but the orientation of N₃GTP and the flexibility of the N-terminal region must be such that this domain can approach the 8-position of the guanine ring.

The data presented here demonstrate a specific GTP binding site on glucagon that may be involved in mechanisms of action of this hormone. The specificity was demonstrated by saturation of labeling with [γ - 32 P]8N₃GTP and protection with the natural nucleotide at physiolog-

Table 3. Amino acid sequence analysis of radiolabeled tryptic and chymotryptic peptides from photolabeled glucagon

Cycle	Identified amino acid	Radiolabeled tryptic peptide (pmol)	Radiolabeled chymotryptic peptide (pmol)
1	His	304	35.1
2	Ser	354	16.4
3	Gln	506	36.9
4	Gly	400	11.1
5	Thr	271	9.5
6	Phe	346	7.1
7	Thr	203	
8	Ser	113	
9	Asp	143	
10	Tyr	120	
11	Ser	93	
12	Lys	54	

ically relevant concentrations. The intracellular concentration of GTP is in the 0.2 mM range, and we observed binding affinities in the low micromolar range. Labeling was greatest at pH values between 5.0 and 6.5, which is near the pH values in the endosome or intracellular matrix, respectively. Decreased photoinjection was observed at pH values above 7.0. This may be due to pH-induced conformational changes of glucagon that change the affinity for GTP or that place the azido group in a less favorable position for photoinjection. This possibility is supported by other studies where distinct changes in the structure of glucagon are observed when the pH is lowered to the acidic range (Braun et al., 1983; Oomen & Kaplan, 1990). Glucagon is reportedly more likely to be in a monomeric conformation under acidic and alkaline conditions, whereas at neutral pH it tends to be in a trimeric form (Boesch et al., 1978). Such a pH-dependent conformational modulation may allow glucagon to bind GTP with greater affinity at the more acidic pHs, which may be the conditions the hormone encounters inside the cell, especially within the endosome. Because the pK_a value of histidine is about 6.4, the protonated form of this N-terminal residue may enhance photolabeling, as no significant photoinjection was observed at pH values above 7.0.

The GTP-glucagon interaction could affect the activity of the hormone by preventing its processing by endogenous proteases or by changing the affinity of glucagon for its receptor as it is internalized. The observed 8–12 μ M K_d of glucagon for GTP suggests a physiological role for this hormone-nucleotide interaction. This affinity is near the half-maximal GTP-mediated inhibition of processing of the hormone bound to the receptor of canine hepatic membranes (Sheetz & Tager, 1988a). Although the pH in those studies was 7–8, the optimal pH for this enzyme in the studies ranged from 5 to 9.

The ATP interactive site detected using [γ - 32 P]8N₃ATP indicates that ATP may possibly be involved in glucagon-mediated events. However, GTP protected this site from [γ - 32 P]8N₃ATP photoinjection better than ATP and with the same K_d that it demonstrated with [32 P]8N₃GTP. This suggests that this is the same site labeled by [32 P]-8N₃GTP.

The N-terminal region of glucagon corresponding to the first six amino acid residues (HSQGTF) was found to be the modified peptide region. This was confirmed with proteolytic digests using either chymotrypsin or trypsin. According to other studies, the N-terminal region may be important for biological activity through a mechanism not involving receptor binding (Gefel et al., 1990; Mommsen & Moon, 1990; Plisetskaya, 1990). Some studies have suggested that the N-terminal region of the hormone forms an aliphatic α -helix, which inserts into the lipid bilayer (Oomen & Kaplan, 1990). However, a study using 2-D NMR and laser photochemically induced dynamic nuclear polarization NMR has suggested that

the N-terminal region of glucagon is not responsible for binding to lipid vesicles and that this region's milieu is unchanged even after binding to lipid micelles (Braun et al., 1983).

Mutations have been introduced into glucagon at Asp-9, which greatly reduce the adenylate cyclase activation by the hormone upon incubation with rat liver membranes. However, the receptor-binding affinities of these glucagon analogs were relatively unaffected (Unson et al., 1991). The Asp-9 residue may be important for nucleotide binding, as the interaction between this residue and His-1 may form a pocket to accommodate a nucleotide-binding site. Alternatively, Asp-9 may form a salt bridge with Lys-12, which would keep glucagon in a conformation favoring an interaction with GTP.

The importance of the N-terminal region is supported by results obtained using a glucagon-like peptide (GLP-1). GLP-1 is an alternate splice product containing amino acid residues 7–37 of the preproglucagon gene. GLP-1 is able to bind to the receptors with the same affinity as glucagon, yet able to elicit only a 2-fold increase in cAMP production over basal compared to a 20-fold glucagon-induced increase (Mommsen & Moon, 1990; Plisetskaya, 1990). Another study showed that an analog of glucagon, where His-1 was 3-methylated and Lys-12 was mutated to Arg, binds to the receptor twice as well as glucagon. This analog was five times less effective in stimulating adenylate cyclase activity (McKee et al., 1986). In these and other studies the N-terminal region seems to be important for modulating activity rather than affecting hormone binding to the receptor. It is quite likely that GTP binding is disrupted with these glucagon analogs and that the GTP-glucagon complex plays an important role in the activation of adenylate cyclase.

Although the definite mechanistic involvement and the cellular location of the glucagon-GTP interaction is unclear, the combined data from several laboratories seem to support its importance. We propose that glucagon analogs lacking intact N-terminal regions, which contain at least part of the guanosine binding domain, may serve primarily as antagonists by mimicking binding to the receptor. However, they cannot induce maximum adenylate cyclase activity due to their inability to form the proper GTP-glucagon complex.

The physiological relevance of nucleotide interactions with glucagon or other peptide hormones has yet to be determined. However, in preliminary investigations in our laboratory with IL-2 and granulocyte-macrophage colony-stimulating factor, biological activity is greatly reduced from control levels on modification with the appropriate nucleotide photoprobe. These biological response modifiers showed sequence homology to several nucleotide binding proteins with the IL-2 photolabeled peptide having high homology with a domain in the pertussis toxin catalytic subunit that has been suggested to represent part of the ADP-ribosyltransferase site. Efforts

to test whether glucagon modified by $8N_3GTP$ is able to compete with glucagon for binding to the receptor are currently underway. Similarly, adenylate cyclase activation by $8N_3GTP$ -modified glucagon will need to be tested and compared to that of glucagon in order to definitively determine the effect on biological activity due to nucleotide-hormone interaction. It is likely that GTP binding to glucagon confers some relevant physiological change with biological significance and represents another aspect in biological response modifier and polypeptide hormone-mediated events that must be considered when detailing the mechanism of action of such biomolecules.

Materials and methods

$[\gamma^{32}P]8N_3GTP$, $[\gamma^{32}P]8N_3ATP$ (specific activity 11–28 mCi/ μ mol), and $[\alpha^{32}P]8N_3GTP$ were prepared and purified as previously reported (Geahlen & Haley, 1977; Potter & Haley, 1983). Molecular weight standards were obtained from Bio-Rad. Glucagon and all other reagents were obtained from Sigma Chemical Company and were analytical grade.

Photolabeling of glucagon

For saturation studies, 1 μ g of glucagon in 60 μ L of reaction buffer (22.5 mM MES, pH 5.5) was incubated in Eppendorf tubes with the particular photoprobe for 10 s and then irradiated with a hand-held 254-nm Mineralight UVS-11 UV lamp ($I = 4,600 \mu W/cm^2$) for 60 s at room temperature. The reaction was quenched with 20 μ L of a protein solubilizing mix containing 0.125 M Tris-HCl, pH 6.8, 0.2% SDS, 50% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue according to Schagger and Von Jagow (1987) and analyzed by SDS-PAGE.

For protection studies, glucagon was incubated with a competitor for 10 s prior to the addition of photoprobe. The reaction solution was photolabeled, quenched, and analyzed by SDS-PAGE as described above.

In the multiple photolyses studies for HPLC analyses, 100 μ g of glucagon were incubated with saturating amounts (40 μ M; specific activity 11–28 mCi/ μ mol) of $[\gamma^{32}P]8N_3GTP$ at pH 5.5 and photolabeled. Nonradioactive $8N_3GTP$ was then added to a final concentration of 40 μ M and the sample photolabeled again. The reaction solution was then prepared for enzymatic digestion. When analyzing for total incorporation at each step, $[\gamma^{32}P]8N_3GTP$ was used during the second and third incubations instead of $8N_3GTP$. After each photolabeling, equal amounts of protein were aliquoted, quenched, and separated on SDS-PAGE.

SDS-PAGE and scintillation

Photolabeled samples were subjected to SDS-PAGE in 3% stacking, 8% spacing, and 20% separating gels as de-

scribed previously (Schagger & Von Jagow, 1987). The samples were electrophoresed at a constant 30 V until all of the tracking dye had run into the stacker and then electrophoresed at a constant 100 V for about 16 h. After electrophoresis, the gels were fixed in (40:7:5:48) MeOH:HOAc:glycerol:H₂O for an hour, dried on a slab gel dryer, and subjected to autoradiography for 2–5 h. ^{32}P incorporation into glucagon on gels was determined by excision of the gels followed by scintillation counting in a Packard Minaxi scintillation counter (99% efficient).

Enzymatic digestion and separation and sequencing of photolabeled peptides

Photolabeled samples were subjected to enzymatic digestion by incubation with 0.5% (w/w) of either trypsin or chymotrypsin for 2 h at pH 8.0 in 100 mM ammonium bicarbonate at room temperature. The entire sample was concentrated for 2–4 h on a Savant Speedvac concentrator. The samples were then subjected to reverse-phase HPLC using an aquapore RP 300 C₈ column (Brownlee Laboratory) on an LKB HPLC system equipped with a diode array spectral detector. The mobile system consisted of a 0.1% trifluoroacetic acid TFA (A) and 0.1% TFA/70% acetonitrile (B) solvent system. The gradient for HPLC was 0–10 min, 0% B; and, 10–70 min, 0–100% B at a flow rate of 0.5 mL/min. When a second HPLC run was done to confirm comigration of peptide and radiolabel, the following gradient was used: 0–10 min, 0% B; 10–15 min, 0–25% B; 15–20 min, 25% B; 20–60 min, 25–40% B; and, 60–70 min, 40–100% B. HPLC fractions containing photolabeled peptides were sequenced on an Applied Biosystem 677A protein sequencer with an on-line phenylthiohydantoin identification.

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