Importance of the 10–13 Region of Glucagon for Its Receptor Interactions and Activation of Adenylate Cyclase†

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ABSTRACT: The role of the Tyr[10]-Ser[11]-Lys[12]-Tyr[13] region of glucagon in the binding interaction and activation of the glucagon receptor was investigated by means of the synthetic glucagon analogues [Phe[13]]glucagonamide (**2**), [Phe[10]]glucagonamide (**3**), [Phe[10]]glucagon (**4**), [Phe[10,13]]glucagon (**5**), [Pro[11]]glucagon (**6**), [Pro[11],Gly[12]]glucagonamide (**7**), [Ala[11]]glucagon (**8**), and [Oac[11-13]]glucagonamide (**9**). These analogues were synthesized by solid-phase peptide synthesis on *p*-methylbenzhydrylamine or Merrifield resins with protected N^{α}-*tert*-butyloxycarbonyl amino acids. Purification by dialysis, cation-exchange chromatography, gel filtration, and preparative reverse-phase high-performance liquid chromatography (HPLC) gave products that proved homogeneous by thin-layer chromatography and HPLC and on analysis by amino acid analysis, by sequencing, and by α-chymotryptic peptide mapping with HPLC. Biological activities were examined by measurement of the stimulation of liver plasma membrane adenylate cyclase and by specific displacement of [^{125}I]glucagon from glucagon receptors. The results of these studies indicate that while the biological "message" region of glucagon is located elsewhere, the 10–13 region has multiple roles in the glucagon–glucagon receptor interaction: (1) this region provides functional groups for direct binding interaction with the receptor, and (2) this region interacts with the receptor in such a way as to allow the "transduction message" portion of glucagon to interact and activate the receptor.

The 10–13 region of the pancreatic hormone glucagon (**1**) (Figure 1) has been actively studied for its role in glucagon's receptor interaction by means of semisynthetic modifications of glucagon. This work has recently been reviewed (Hruby et al., 1985; Bromer, 1983). Nitration or iodination of either or both of the tyrosine residues of glucagon increases the ability of the hormone to release glucose in rabbits (Patterson & Bromer, 1973; Bromer et al., 1973). However, amination of the tyrosine residues results in a loss of potency. Lin et al. (1976) have proposed that the iodination increases the hydrophobic interaction of the tyrosine residues with the receptor and have demonstrated that ionization of the phenolic groups results in a reduction in binding potency. The tyrosine at position 10 has a concentration-dependent pK_a (Frank & Pekar, 1974). In dilute solution, the pK_a = 9.7 but rises to a value of 10.1 in concentrated solutions. The pK_a of the Tyr[13] residue has a concentration-independent value of 10.4. This finding reversed earlier suggestions of Gratzer and Beaven (1969) and led Korn and Ottensmeyer (1983) to propose that the lowered pK_a of the Tyr[10] residue could be due to its interacting in an intramolecular hydrogen bond. Since CD[1] studies show that the conformation of glucagon is concentration-dependent due to aggregation of the glucagon molecules (Gratzer et al., 1967; Gratzer & Beaven, 1979; Srere & Brooks, 1969), the pK_a dependence on concentration of the Tyr[10] residue could be due to either intra- or intermolecular interactions. The crystal structure of glucagon crystallized at alkaline pH determined by X-ray analysis is a largely α-helical trimeric structure (Sasaki et al., 1975). One of the sites of association in the trimer is between the side chains of Trp[25], Leu[26], and Phe[22] of one molecule with the Tyr[10], Tyr[13], and Phe[6] side chains of another molecule. Thus, intermolecular interaction upon association would be an alternate explanation for the concentration dependence of the Tyr[10] pK_a but does not explain its relatively low value in dilute solutions. In the work reported here, we have further investigated the importance of the phenolic hydroxyl groups of the Tyr residues by substituting one or both of them with a Phe or by removing the side-chain group.

Glucagon analogues modified at the Lys[12] position have demonstrated the importance of a positively charged side chain at this residue. Acylation and other modifications of the ϵ-amino group of Lys[12], which neutralize the charge, yield partial agonists with diminished adenylate cyclase potency (Bregman et al., 1978, 1980; Carrey & Epand, 1982). Des-His[1]-[N^{ϵ}-(phenylthiocarbamoyl)-Lys[12]]glucagon exhibited 15% maximal activation of adenylate cyclase relative to glucagon, whereas des-His[1]-glucagon possesses the ability to stimulate adenylate cyclase activity to 56% of the maximum level obtained with glucagon (Bregman & Hruby, 1979). N^{α},N^{ϵ}-Dicarbamoylglucagon is a partial agonist with 17% maximal activation of adenylate cyclase relative to glucagon and with a 13th of the potency of N^{ϵ}-carbamoylglucagon, which exhibits 27% maximal adenylate cyclase stimulation (Bregman et al., 1980). Analogues that retain the positive charge retain full

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; Bzl, benzyl; cAMP, adenosine cyclic 3′,5′-monophosphate; CD, circular dichroism; Chx, cyclohexyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; EDTA, ethylenediaminetetraacetic acid; For, formyl; GTP, guanosine 5′-triphosphate; HF, hydrofluoric acid; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; Oac, ω-aminocaprylic acid, 8-aminooctanoic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HArg, homoarginine.

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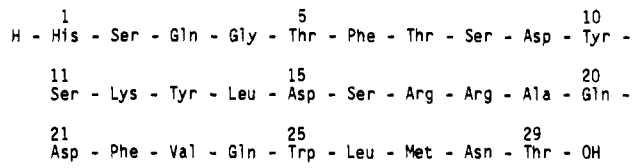

FIGURE 1: Amino acid sequence of glucagon (**1**).

activity though potency may be reduced. [HArg^{12}]glucagon (Bregman et al., 1980; Ross et al., 1979; Hruby et al., 1981), [N^{ϵ}-acetamidino-Lys^{12}]glucagon (Flanders et al., 1982; Wright & Rodbell, 1980), and [N^{ϵ}-[(4-hydroxyphenyl)amidino]-Lys^{12}]glucagon (Wright & Rodbell, 1980) are all full agonists in the adenylate cyclase assay. We have further investigated the importance of the Lys^{12} side chain in this study by replacing it with a methyl group in analogue **8** or by completely eliminating it in our Oac$^{11-13}$ analogue **9** and in analogue **7**.

Chou–Fasman calculations (Chou & Fasman, 1975) on glucagon suggest that there is a potential for the formation of a β-turn centered in the 10–13 region. The X-ray crystallographic structure of glucagon reported by Sasaki et al. (1975) is α-helical for the 10–25 region extended at both ends with a less regular right-handed helix for another four residues. Braun et al. (1983) have reported an α-helix-like turn for the 10–14 region of glucagon from their NMR studies on glucagon in a lipid–water interphase. The importance or existence of a turn in the 10–13 region to the biological activity of glucagon is not known. In the studies reported here, we have chosen analogues that increase or decrease β-turn potential as measured by Chou–Fasman calculations where possible.

In order to accomplish the above goals, it was necessary to prepare totally synthetic glucagon analogues. Therefore, we have prepared a series of totally synthetic glucagon analogues in order to examine the role of this important region of glucagon (residues 10–13) in receptor interaction and activation. In a few cases, glucagonamide analogues were prepared to examine the effect of terminating the C-terminal residue in glucagon as a carboxamide rather than as a carboxylate on the biological activities of glucagon derivatives.

Experimental Procedures

Materials. The following were obtained from commercial sources: Boc-amino acids (Vega Biotechnologies or prepared in our laboratory by standard methods); Boc-Asp(Chx) (Peptides International); trifluoroacetic acid (Halocarbon Products); anisole, ethanedithiol, 1-hydroxybenzotriazole, *N*-acetylimidazole, picric acid, *n*-butyllithium, hexamethyldisilizane, trimethylsilyl chloride, sodium hydride, *p*-methyltolualdehyde, and diisopropylethylamine (Aldrich); 3 N mercaptoethanesulfonic acid, ninhydrin, and Spectra-Por 6 dialysis tubing (Pierce Chemical Co.); chloromethylated polystyrene resin 1% cross-linked with divinylbenzene (Lab Systems); polystyrene resin 1% cross-linked with divinylbenzene and Dowex AG 50W-4 cation exchange resin (Bio-Rad); purified acetonitrile (Burdick & Jackson); acetic acid, urea, ethanol, and methylene chloride (J. T. Baker); Sephadex G-15, SP-Sephadex C-25, and QAE-Sephadex A-25 (Pharmacia); sodium metabisulfite (Mallinckrodt); α-chymotrypsin, chloramine-T, Tris-HCl (reagent grade), bovine serum albumin (fraction v), chromatographic alumina (type WN_3, neutral), cAMP, ATP, GTP, EDTA, phosphocreatine, and creatine phosphokinase (Sigma); carrier-free Na^{125}I in 0.1 N NaOH and ACS scintillation fluid (Amersham); [^{3}H]cAMP (New England Nuclear); [α-^{32}P]ATP (ICN); crystalline porcine glucagon (Elanco); Oxoid cellulose acetate filters (Oxoid U.S.A.); male Sprague-Dawley rats (University of Arizona, Division of Animal Resources).

Resins for Synthesis. Glucagonamide analogues were synthesized on *p*-methylbenzhydrylamine resin, which was prepared by the method of Hirao et al. (1983) from 200–400-mesh Bio-Beads. Resin substitution was determined by the picric acid test (Gisin, 1972) to be 0.7 mmol of amine/g of resin. Boc-Thr(Bzl) (3 equiv) was coupled to the resin as the preformed symmetrical anhydride (Hagenmaier & Frank, 1972) for 30 min. The resin was washed with methylene chloride and treated with *N*-acetylimidazole (3 equiv) in methylene chloride for 5 h in order to acetylate any free amino groups remaining on the resin. Resin substitution was found to be 0.6 mmol of amine/g of resin by the picric acid test and by amino acid analysis.

Glucagon analogues with a free C-terminal carboxyl group were synthesized on Merrifield resin. Coupling of Boc-Thr(Bzl) to chloromethylated polystyrene resin (0.7 mmol of Cl/g of resin) by the method of Gisin (1973) yielded 0.5 mmol of amine/g of resin as determined by the picric acid test and by amino acid analysis for threonine.

Synthesis Methodology. Boc-Asn and Boc-Gln were coupled as their *p*-nitrophenyl esters in the presence of an equimolar amount of 1-hydroxybenzotriazole (HOBT). Boc-Arg(Tos) was coupled with dicyclohexylcarbodiimide (DCC) in methylene chloride/dimethylformamide with an equimolar amount of 1-hydroxybenzotriazole.

The remainder of the Boc-protected amino acids were coupled by the preformed symmetrical anhydride method (Hagenmaier & Frank, 1972). Three equivalents of Boc-amino acid was dissolved in methylene chloride at 0 °C. A total of 1.5 equiv of DCC was added to the solution, and the mixture was kept at 0 °C for 30 min. The solution was filtered of the precipitated dicyclohexylurea and added to the N^{α}-deprotected peptide resin for coupling. The following side-chain protecting groups were utilized: Asp(Chx), Ser(Bzl), Thr(Bzl), His(Tos), Trp(For), Lys(2,6-diClZ), and Tyr(2-BrZ). The deblocking and coupling sequence, which was performed on either a Vega Model 1000 or a Vega Model 250 peptide synthesizer, was as follows: (a) 1 × 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) with 2% anisole for 2 min; (b) 1 × 50% TFA in DCM with 2% anisole for 20 min; (c) three washes with DCM for 2 min; (d) 3 × 10% diisopropylethylamine in DCM for 2 min; (e) three washes with DCM for 2 min; coupling by preformed symmetrical anhydride for 30 min (couplings by DCC/HOBT for 3 h and by active ester for 6 h); (f) 3 × DCM for 2 min; (g) three washes with 100% ethanol for 2 min; (h) three washes with DCM for 2 min. The ninhydrin test was used to monitor the reaction (Kaiser et al., 1970). The coupling was repeated if the reaction was not complete. If the repeated coupling was unsuccessful, then the remaining amino groups were acetylated with *N*-acetylimidazole (3 equiv) in DCM.

Cleavage, Deprotection, Purification, and Characterization. The method of Matsueda (1982) was used for HF deprotection and cleavage of the peptide from the resin. After removal of the HF, the resin was mixed with ethyl ether and filtered. The resin was washed with 30% aqueous acetic acid and the ether filtrate was extracted with 30% acetic acid. The combined washings were lyophilized. The residue was dissolved in the minimum amount of 30% aqueous acetic acid and dialyzed against 2% aqueous acetic acid in Spectra-Por 6 dialysis tubing with a molecular weight cut-off of 1000. The contents of the dialysis tube were lyophilized, and the residue was eluted on a SP-Sephadex C-25 ion exchange column (1 × 25 cm) with a linear gradient of 0–1 N sodium chloride in 6 M urea in 10%

Table I: Amino Acid Analysis of Glucagon Analogues[a]

amino acid	2[b]	3[b]	4[b]	5[b]	6[b]	7[b]	8[b]	9[b]
Trp	1.01 (1)	0.72 (1)	0.96 (1)	0.92 (1)	0.82 (1)	1.01 (1)	0.94 (1)	1.01 (1)
Lys	1.04 (1)	1.05 (1)	0.96 (1)	1.06 (1)	1.01 (1)		1.02 (1)	
His	0.96 (1)	0.92 (1)	0.94 (1)	1.10 (1)	0.98 (1)	1.08 (1)	0.98 (1)	1.09 (1)
Arg	2.00 (2)	2.03 (2)	2.12 (2)	1.98 (2)	2.21 (2)	1.90 (2)	2.07 (2)	1.90 (2)
Asx	4.08 (4)	3.91 (4)	4.29 (4)	3.84 (4)	4.20 (4)	4.24 (4)	4.16 (4)	3.73 (4)
Thr	2.97 (3)	2.81 (3)	2.71 (3)	2.87 (3)	2.80 (3)	2.97 (3)	3.03 (3)	2.74 (3)
Ser	3.87 (4)	3.76 (4)	4.04 (4)	3.50 (4)	2.93 (3)	2.76 (3)	2.70 (3)	3.00 (3)
Glx	3.00 (3)	2.92 (3)	3.18 (3)	2.91 (3)	2.86 (3)	3.16 (3)	3.08 (3)	2.92 (3)
Pro					1.04 (1)	1.05 (1)		
Gly	1.02 (1)	1.05 (1)	1.04 (1)	0.92 (1)	1.05 (1)	2.04 (2)	1.17 (1)	0.98 (1)
Ala	1.03 (1)	1.01 (1)	1.15 (1)	0.95 (1)	1.00 (1)	1.01 (1)	2.00 (2)	0.94 (1)
Val	0.99 (1)	1.01 (1)	1.15 (1)	1.04 (1)	0.98 (1)	1.05 (1)	1.13 (1)	0.98 (1)
Met	1.02 (1)	0.95 (1)	1.07 (1)	1.00 (1)	0.98 (1)	1.00 (1)	1.01 (1)	1.07 (1)
Leu	2.02 (2)	2.03 (2)	2.09 (2)	2.06 (2)	2.10 (2)	2.02 (2)	1.98 (2)	2.02 (2)
Tyr	1.08 (1)	1.07 (1)	0.97 (1)		2.08 (2)	1.88 (2)	1.92 (2)	1.01 (1)
Phe	2.93 (3)	2.86 (3)	2.95 (3)	4.27 (4)	2.05 (2)	2.01 (2)	1.90 (2)	2.01 (2)

[a] Hydrolyses were performed for 48 h at 110 °C in 6 N HCl with 0.2% phenol and for 24 h at 110 °C in 3 N mercaptoethanesulfonic acid. [b] The numbered glucagon analogues are as follows: **2**, [Phe[13]]glucagonamide; **3**, [Phe[10]]glucagonamide; **4**, [Phe[10]]glucagon; **5**, [Phe[10,13]]glucagon; **6**, [Pro[11]]glucagon; **7**, [Pro[11],Gly[12]]glucagonamide; **8**, [Ala[11]]glucagon; **9**, [Oac[11-13]]glucagonamide.

Table II: Physical Data for Glucagon Analogues

no.	compd	M_r[a]	ϵ_{279}[b]	yield (%)[c]	TLC, R_f[d] I	TLC, R_f[d] II	HPLC, k'[e]
2	[Phe[13]]glucagonamide	3465.87	5373	1.1	0.60	0.64	9.84
3	[Phe[10]]glucagonamide	3465.87	5998	1.6	0.62	0.66	15.76
4	[Phe[10]]glucagon	3466.85	6334	10.0	0.68	0.67	16.94
5	[Phe[10,13]]glucagon	3450.85	5320	0.6	0.69	0.62	11.98
6	[Pro[11]]glucagon	3492.87	7104	0.9	0.70	0.66	1.38
7	[Pro[11], Gly[12]]glucagonamide	3420.81	6562	0.5	0.62	0.64	10.24
8	[Ala[11]]glucagon	3266.85	6772	5.5	0.66	0.65	6.89
9	[Oac[11-13]]glucagonamide	3244.89	6116	3.0	0.63	0.65	16.16

[a] Calculated. [b] Determined from the amino acid analysis (glucagon ϵ_{280} = 8310). [c] Yield (%) of purified compound based on the initial resin substitution. [d] TLC: silica gel 60 F-245, 5 × 20 glass plates, 0.25-mm layer thickness (Merck catalog no. 57613). R_f for glucagon in solvent system I is 0.65 and in solvent system II is 0.63. Solvent systems: I, 1-butanol–acetic acid–water–pyridine (60:12:48:60); II, 2-propanol–ammonium hydroxide–water (3:1:1). [e] Vydac 218TPB-16 C18 column (4.6 × 250 mm), 2 mL/min, 30% CH_3CN in 0.1% TFA, void volume = 3.4 mL (k' for glucagon = 6.49).

aqueous acetic acid (pH 3.3). The major peak, usually eluting with an approximately 0.3 N chloride concentration, was collected and eluted on a Sephadex G-15 column (25 × 90 mm) with 5% aqueous acetic acid. The peak eluting with the void volume was collected and lyophilized. The residue was eluted on a Perkin-Elmer preparative HCODS column (25 × 250 mm) with 33–35% acetonitrile in 0.1% aqueous TFA over 20 min at 10 mL/min. The eluant corresponding to the major component (detected by UV adsorption at 279 nm) was collected and lyophilized. Figure 2 depicts the HPLC analysis of [Phe[13]]glucagonamide at various stages of the purification scheme. Plate a is after HF cleavage and deprotection and dialysis. Plate b is after ion exchange chromatography and desalting. Plate c is after preparative HPLC.

Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer (Table I). Sample hydrolysis was done for 48 h at 110 °C in 6 N hydrochloric acid with 0.2% phenol and for 24 h at 110 °C in 3 N mercaptoethanesulfonic acid. Homogeneity was determined by TLC and by HPLC on a Vydac 218TPB-16 C18 column (4.6 × 250 mm) (Table II). Peptide sequencing of [Phe[13]]glucagonamide was performed on a Beckman Model 890 M peptide sequencer, and this confirmed the correct sequence. Further confirmation of structure was obtained by peptide mapping with α-chymotrypsin (Table III).

The chymotryptic mapping was performed as follows. The peptide analogue (0.5 mg) was dissolved in 0.5 mL of a solution of 2.3 mg of α-chymotrypsin in 100 ml of 10 mM sodium bicarbonate buffer (pH 8.1). The mixture was incubated at 30 °C for 16 h. Another 0.5 mL of the enzyme solution was added and the mixture incubated at 30 °C for another 11 h. The mixture was lyophilized. The residue was analyzed by HPLC on a Vydac 218TPB-16 column (4.6 × 250 mm). With a 3–33% acetonitrile gradient in 0.1% TFA over 30 min at 2 mL/min, the retention times of the peaks eluting were noted and the peaks identified by amino acid analysis (Table III).

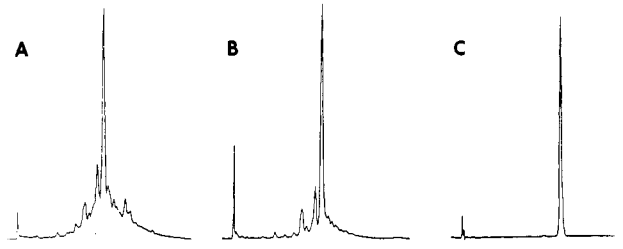

FIGURE 2: HPLC analysis of [Phe[13]]glucagonamide at various stages of purification: (A) after HF deprotection and cleavage followed by dialysis; (B) after cation-exchange chromatography and gel filtration; (C) after preparative high-performance liquid chromatography. These chromatograms represent a 25–50% gradient of acetonitrile in 0.1% TFA over 25 min at 2 mL/min on a Vydac 218TPB-16 C18 column (4.6 × 250 mm) monitored at 279 nm.

The use of peptide mapping allows verification of structure. Those portions of the glucagon molecule that are unchanged fragment in a pattern identical with that of glucagon, and those portions that are modified show up as new components, relative to the glucagon map, which can be isolated and identified. α-Chymotrypsin was chosen because it degrades glucagon primarily into seven fragments, the largest being the N-terminal hexapeptide. Trypsin, in theory, would yield only three

Table III: α-Chymotryptic Peptide Mapping of Glucagon Analogues[a]

no.	compd	1–6	7–10	11–13	7–13	14–22	7–17	18–22	23–25	26–29
1	glucagon	12.3	8.4	7.4	12.9	16.0		14.2	18.5	11.0
2	[Phe^{13}]glucagonamide	12.2	8.4	10.7	16.5	16.0		14.1	18.4	9.1
3	[Phe^{10}]glucagonamide	12.4		7.2	16.0	16.0		14.3	18.6	9.2
4	[Phe^{10}]glucagon	12.3		7.5	16.5	15.9		14.1	18.5	11.0
5	[Phe^{10,13}]glucagon	12.2		10.7	20.1	16.4		14.2	18.8	10.7
6	[Pro^{11}]glucagon	12.6			20.6	16.4		14.0	18.8	11.3
7	[Pro^{11},Gly^{12}]glucagonamide	12.3			21.0	16.2		14.0	18.7	9.1
8	[Ala^{11}]glucagon	12.4	8.4	8.1		16.2		14.2	18.6	11.1
9	[Oac$^{11-13}$]glucagonamide	12.3					28.2	14.0	18.6	9.2

[a] The values given are the retention times in minutes for the listed fragments. The peptides maps are produced by elution on a Vydac 218TPB-16 C18 column (4.6 × 250 mm) with a 3–33% acetonitrile gradient in 0.1% aqueous trifluoroacetic acid over 30 min at 2 mL/min, monitoring at 214 nm. The identity of the fragments was determined by amino acid analysis. The 23–25 fragment was also shown to be Erlich-positive, confirming the presence of tryptophan.

relatively large fragments that would not define the position of substitution as well as the fragmentation provided by α-chymotrypsin.

Biological Methods. Plasma membranes were prepared from liver of male Sprague-Dawley rats weighing 140–180 g according to the procedure of Neville (1968) modified by Pohl et al. (1971). Amount of protein was determined by the method of Markwell et al. (1978) and 1–2-mg protein aliquots in 25 mM Tris buffer, pH 7.5, were stored in liquid N_2 for 2–3 months before using them for adenylate cyclase and receptor binding assays.

Glucagon and synthetic glucagon analogues were dissolved in 2 mM Na_2CO_3 to a concentration of 100 μM and further diluted by 25 mM Tris buffer (pH 7.5 at 25 °C) containing 1% BSA to a suitable concentratin range for adenylate cyclase and receptor binding assays. Peptides were stored at −20 °C in the form of lyophilized powders.

Adenylate Cyclase Assay. Adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to cyclic 3′,5′-AMP as described by Lin et al. (1975). Furthermore, labeled cAMP was determined by the method of Salomon et al. (1976) using Dowex 50 and alumina chromatography. Briefly, 0.1 mL of incubation medium consists of 1 mM [α-^{32}P]ATP (about 50 cpm/pmol), 5 mM $MgCl_2$, 10 mM GTP, 1 mM EDTA, 1 mM cAMP containing 10 000 cpm of [^{3}H]cAMP, 25 mM Tris-HCl, pH 7.5 at 25 °C, 1% BSA, 35 μg of membrane protein, and an ATP regenerating system that had 20 mM phosphocreatine and 0.72 mg/mL (100 units/mL) creatine phosphokinase. Results are expressed as a potency relative to glucagon (defined as 100) and in terms of the maximal stimulation of adenylate cyclase by glucagon (which is defined as 100%) or analogue under these assay conditions.

Receptor Binding Assay. Radioiodination of glucagon was done by the method of Hagopian and Tager (1983). A 1.0-nmol sample of carrier-free Na^{125}I (2.0 mCi) was allowed to react with 3.0 nmol of glucagon in the presence of 1.5 nmol of choramine T, added at a regular interval of 30 s (0.5 nmol each time). Sodium metabisulfite solution (0.5%) was used to stop the reaction, followed by 1 mL of elution buffer containing 0.08 M Tris, 0.02 M HCl, 0.08 M NaCl, and 1% BSA. QAE-Sephadex A-25 was employed for chromatographic purification of the labeled glucagon as described by Jorgensen and Larson (1972). Two-milliliter fractions were collected, and the monoiodinated glucagon peak, as determined by reverse-phase HPLC, was stored at −20 °C for use within 2 weeks for receptor binding assay following the method of Lin et al. (1975).

A sample of liver plasma membrane containing 50 μg of protein, 120 000 cpm of [^{125}I]glucagon, and unlabeled glucagon or glucagon analogues at desired concentrations (range 0–10 μM) was incubated for 10 min at 30 °C. The final volume of the incubation medium was kept at 500 μL with the aid of 25 mM Tris-HCl (pH 7.5) containing 1% BSA. After the incubation mixture was cooled for 15 min, 0.45-μm Oxoid filters, soaked overnight in Tris–BSA buffer, were used to filter it immediately. Four milliliters of ice-cold 25 mM Tris-HCl buffer, pH 7.5, was then used for washing, and the amount of radioactivity remaining on filters was quantitated by an LKB 1275 mini gamma counter. Nonspecific binding was found to be typically 15–20%, and correction was applied to calculate specific binding. Results are expressed as the percent inhibition of [^{125}I]glucagon specific binding.

In both the assays, triplicate determination of each data point was obtained, and all experiments were carried out at least twice. The EC_{50} values and 95% confidence limits for each of the compounds in each assay were determined by the statistical methods given by Bowman and Rand (1980).

Results and Discussion

Solid-phase synthesis of the glucagon analogues reported here proceeds smoothly on *p*-methylbenzhydrylamine resins or standard chloromethylated Merrifield resins depending on whether the carboxamide-terminal glucagon analogues or the carboxylate-terminal glucagon analogues were desired (see Experimental Procedures). Generally, coupling to the growing peptide chain was accomplished by the symmetrical anhydride method, though Boc-Asn and Boc-Gln were coupled as their *p*-nitrophenyl esters with 1-hydroxybenzotriazole catalysis and Boc-Arg(Tos) was coupled by the DCC/HOBt method. After cleavage of the peptide from the resin and removal of the side-chain protecting groups, both of which were accomplished by the HF procedure used (see Experimental Procedures), the glucagon analogues were purified by a combination of dialysis, gel filtration on Sephadex G-15, ion exchange chromatography on SP-Sephadex C-25, and preparative reverse-phase high-performance liquid chromatography (RP-HPLC) with a Perkin-Elmer HCODS column. Efforts to purify the peptides by use of a preparative RP-HPLC alone were unsuccessful, yielding products that were not of satisfactory purity. Final purities were greater than 98% in all cases.

The purity of the final products was assessed by amino acid analysis following hydrolysis by two different methods, by thin-layer chromatography, and by enzymatic peptide mapping with RP-HPLC followed by amino acid analysis. Highly satisfactory results were obtained in all cases (see Experimental Procedures, Tables I–III, and Figure 2). Particularly useful were the peptide mapping procedures we have developed using α-chymotrypsin digestion followed by RP-HPLC and amino acid analysis. As seen in Table III, the HPLC pattern when combined with amino acid analysis for final proof of structure on the appropriate fragment gave unequivocal confirmation

Table IV: Biological Activities for Glucagon and Glucagon Analogues

		adenylate cyclase		receptor binding	
no.	compd	EC_{50}[a]	relative potency[b]	IC_{50}[c]	relative potency[d]
1	glucagon	8.4 (6.2–11.2)	100 (100)	3.6 (2.8–4.4)	100
2	[Phe^{13}]glucagonamide	10.4 (6.9–15.6)	81 (100)	3.3 (2.9–3.8)	109
3	[Phe^{10}]glucagonamide	7.0 (5.2–9.3)	120 (85)	3.0 (2.5–3.6)	120
4	[Phe^{10}]glucagon	69.8 (52.9–92.2)	12 (84)	19.8 (14.1–27.9)	18
5	[Phe^{10,13}]glucagon	78.2 (63.8–95.9)	11 (89)	35.4 (25.3–49.6)	10
6	[Pro^{11}]glucagon	i.a. at 100 μM		65 400 (56 300–76 000)	0.006
7	[Pro^{11},Gly^{12}]glucagonamide	i.a. of 40 μM		25 000[e]	0.014
8	[Ala^{11}]glucagon	31.4 (24.2–40.8)	27 (40)	15.0 (12.1–18.5)	24
9	[Oac$^{11-13}$]glucagonamide	1980 (1570–2500)	0.4 (100)	1 300 (1 030–1 630)	0.3

[a] EC_{50} (nM) with the 95% confidence limits in parentheses. [b] Relative potency to glucagon = 100 for the linear portion of the dose–response curve. The maximal activation of adenylate cyclase relative to glucagon (=100; 10^{-6} M glucagon gives 100% maximal activation of adenylate cyclase under these assay conditions) is given in parentheses. [c] IC_{50} (nM) with 95% confidence limits in parentheses. [d] Relative potency to glucagon = 100. [e] 52% displacement of iodoglucagon at 25 μM and 96% displacement at 100 μM.

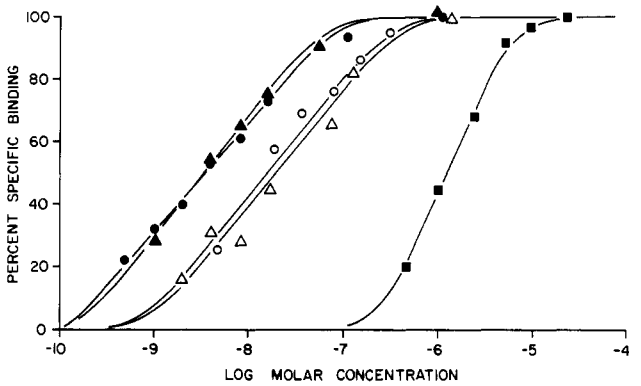

FIGURE 3: Receptor binding data for glucagon (●), [Phe^{13}]-glucagonamide (▲), [Phe^{10}]glucagon (△), [Ala^{11}]glucagon (○), and [Oac$^{11-13}$]glucagonamide (■) as measured from the displacement of [^{125}I]glucagon from rat liver plasma membranes and corrected for nonspecific binding with glucagon as the standard (0 nM = 0% and 1024 nM = 100%).

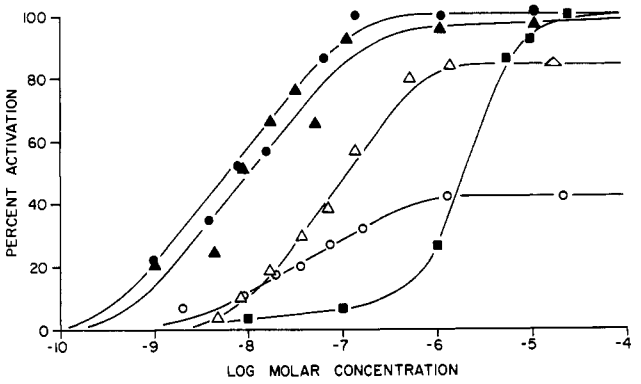

FIGURE 4: Adenylate cyclase activation data for glucagon (●), [Phe^{13}]glucagonamide (▲), [Phe^{10}]glucagon (△), [Ala^{11}]glucagon (○), and [Oac$^{11-13}$]glucagonamide (■) as measured from the production of [^{32}P]cAMP in rat liver plasma membranes with glucagon as the standard (0 nM = 0% and 1024 nM = 100%). The maximal activation of adenylate cyclase by glucagon is about 3-fold over basal levels and is defined as 100%. Cyclase stimulation of analogues is reported as a percentage of the maximal stimulation by glucagon (=100%).

of structure. In the case of the [Phe^{13}]glucagonamide analogue, additional confirmation of structure was obtained by complete sequencing of the analogue on a Beckman Model M peptide sequencer.

Once the highly purified glucagon analogues were available, they were carefully examined for their biological activities. [Phe^{13}]- and [Phe^{10}]glucagonamide (**2** and **3**) both retain adenylate cyclase potency and receptor binding affinity similar to that of glucagon (**1**) although the [Phe^{10}]glucagonamide analogue (**3**) is a partial agonist (Table IV; Figures 3 and 4). [Phe^{10}]glucagon (**4**) is also a partial agonist, giving a maximal activation of liver adenylate cyclase that is 84% that given by glucagon; however, its potency in that system and for receptor binding is diminished relative to the corresponding glucagonamide analogue **3**. Therefore, modifying the C-terminus of glucagon analogues from a carboxylic acid to an amide would appear to be a means of increasing potency without affecting intrinsic activity. [Phe^{10,13}]glucagon (**5**) is a partial agonist much like [Phe^{10}]glucagon but with lower receptor binding potency. Thus, we conclude that while removal of the phenolic group from either of glucagon's tyrosine residues reduces potency of the hormone, for its receptor, only removal of the Tyr^{10} phenolic group results in the inability of the compound to fully activate the receptor complex for maximal adenylate cyclase activity.

Similar to the iodoglucagon analogues (Lin et al., 1976), lipophilicity should be increased by these modifications. Indeed, the retention time on an octadecylsilyl HPLC column is increased for the phenylalanyl analogues (Table II) relative to the tyrosyl-containing derivatives. Since the phenylalanine-containing analogues are less potent, it would appear that the increased potency of the iodinated derivatives is not due solely to the increased overall lipophilicity of the glucagon molecule. The substitution of the tyrosyl residues with phenylalanine serves to decrease the potential for a β-turn in the 10–13 region while enhancing the α-helical potential based on Chou–Fasman calculations (Table V). For example, the $\langle P_a \rangle$ for [Phe^{10,13}]glucagon is increased from 0.827 to 1.047 (a value of 1.03 is considered significant), and the $\langle P_t \rangle$ is significantly decreased from 1.180 to 0.910 (a value of 1.00 is used for predicting a turn). On the basis of NMR studies (Braun et al., 1983) of the conformation of glucagon in the presence of lipid micelles, the 10–14 region possesses an α-

Table V: Chou–Fasman Calculations on the 10–13 Region of Glucagon and Its Analogues[a]

	$\langle P_a \rangle$	$\langle P_b \rangle$	$\langle P_t \rangle$	p_t (×10^4)
glucagon	0.827	1.107	1.180	1.026
analogue				
[Phe^{10}]	0.937	1.084	1.045	0.738
[Phe^{13}]	0.937	1.084	1.045	0.533
[Phe^{10,13}]	1.047	1.062	0.910	0.384
[Pro^{11}]	0.777	0.057	1.202	2.221
[Pro^{11},Gly^{12}]	0.629	1.060	1.340	5.862
[Ala^{11}]	0.990	1.128	0.988	0.561

[a] $\langle P_a \rangle$, $\langle P_b \rangle$, and $\langle P_t \rangle$ are the average conformational parameters for α-helix, β-sheet and β-turn structures, respectively. The value p_t is the relative probability that the tetrapeptide sequence will form a β-turn. All values were determined as described in Chou and Fasman (1974).

helical turn. The nature of the turn in this region is such that a hydrophobic patch is formed by the Phe[6], Tyr[10], and Tyr[13] side chains as in the case of the X-ray crystallographic structure of glucagon (Sasaki et al., 1975). Thus, it would seem, on the basis of the phenylalanyl analogues, that enhancement of an α-helical potential in the 10–13 region is not favorable for receptor interaction. However, the reduced potency of these analogues also could be due to the removal of the phenolic hydroxyl groups if they are involved in the glucagon–receptor interaction.

The introduction of proline at the 11-position to induce a turn in the 10–13 region greatly decreases the receptor binding potency of the hormone. In both [Pro[11]]glucagon and [Pro[11],Gly[12]]glucagonamide (**6** and **7**), no adenylate cyclase activity is detectable even at concentrations where the analogues cause full displacement of [^{125}I]glucagon from liver plasma membrane glucagon receptors. [Ala[11]]glucagon itself is a partial agonist with reduced potency. The fact that Ser[11] appears to have some function in glucagon's activity does not fully account for the loss of activity seen with Pro[11] analogues.

Replacement of the Ser[11], Lys[12], and Tyr[13] residues with ω-aminocaprylic acid [$NH_2(CH_2)_7CO_2H$, 8-aminooctanoic acid, Oac] results in an analogue, **9**, with greatly reduced potency but with the ability to fully stimulate adenylate cyclase. ω-Aminocaprylic acid serves as a spacer group with molecular dimensions similar to those of the backbone of a tripeptide but with greater flexibility and devoid of two amide groups and the side-chain functionalities of the three residues. This finding shows that none of the 11–13 residues are *essential* for transducing the biological message of glucagon, but apparently they are important for maximal binding. Nonetheless, these amino acids serve a greater purpose in the molecule than just providing functional groups for receptor binding potency. Since as we have shown in this work partial agonists can be produced by relatively simple modifications of the Ser[11] and Lys[12] residues, these residues must be important for the ability of the biological activity message (Hruby, 1981) portion of the hormone to interact properly with the receptor. Current structure–activity relationships have led to the conclusion that the N-terminal region of glucagon contains the biological activity message of glucagon (Rodbell et al., 1971; Hruby et al., 1985). This conclusion combined with the results of this paper suggests the following model for the role of the 10–13 region of glucagon in its biological activity. If the 6, 10, and 13 residues are involved in a hydrophobic interaction with the receptor, which is possible on the basis of the appearance of this hydrophobic region in the crystal structure (Sasaki et al., 1975) and the solution conformation (Braun et al., 1983), then the manner of binding of this region of glucagon to the receptor could direct the manner of the interaction of the N-terminal region with the receptor, thereby influencing its ability to fully stimulate the receptor. In view of these results, it is reasonable to suggest that alterations in the manner of binding of the 10–13 region of glucagon to its receptor by (1) modifications of the nature of the lipophilic residues (e.g., Phe for Tyr), (2) modifications of the nature of the hydrophilic residues (e.g., Ala for Ser or Lys), or (3) conformational changes or loss of flexibility (e.g., Phe for Tyr or Pro for Ser) could affect the orientation of the N-terminal region to the receptor and its potential for binding and for activating the adenylate cyclase system (transduction). In the case of [Oac[11-13]]glucagonamide, any binding interactions of the Ser[11], Lys[12], and Tyr[13] residues with the receptor are removed, and their orienting abilities are lost. Thus, the N-terminal region can freely interact with the receptor and fully activate the hormone–receptor complex although potency is greatly reduced with the removal of the binding interactions of the 11–13 region.

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Modulation of the Sensitivity of Chromatin to Exogenous Nucleases: Implications for the Apparent Increased Sensitivity of Transcriptionally Active Genes†

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ABSTRACT: We have examined the effects of changing the ionic composition of the buffers in which nuclei are isolated on the sensitivity of chromatin to micrococcal nuclease and deoxyribonuclease I. Unless nuclei are isolated in buffers containing physiological levels of monovalent (150 mM KCl) and divalent (2–5 mM $MgCl_2$) cations, there is a substantial loss of higher order structure. The ionic composition of the buffer in which the digestion is carried out also affects the amount of material digested both by modulating higher order structure and by determining the solubility of the released material. Magnesium ion concentrations greater than 2 mM and calcium ions at virtually any concentration precipitate substantial amounts of the released chromatin fragments. These observations can be interpreted in light of the known effects of the ions on 10- and 30-nm fiber structure and used as a basis for improvements in techniques for isolating chromatin and for studying its structure and function using exogenous nuclease probes. The apparent nuclease sensitivity of transcriptionally active chromatin was reexamined and shown to be more likely a reflection of differential solubility rather than an overall increase in nuclease sensitivity.

Chromatin is packaged in the nucleus of mammalian cells in a complex series of higher order forms that appear to relate to the functional state of each particular type of cell [for reviews, see Weisbrod (1982), Walker (1983), Butler (1983), and Tsanev (1983)]. Changes in gene expression during adaptation, proliferative activation, and differentiation are generally preceded by alterations in chromatin structure as genes are mobilized from a transcriptionally inactive state to a form that can be transcribed. Consequently, there is considerable interest in the regulatory mechanisms that govern these transitions. Furthermore, when the genetic material is replicated, there are also pronounced structural changes with chromatin becoming decondensed during the S phase of the cell cycle and subsequently becoming highly condensed during late G2 and mitosis.

Exogenous nucleases such as micrococcal nuclease (MNase) and deoxyribonuclease I (DNase I) have been widely used to probe the structure of chromatin in isolated nuclei. For example, they have played a central role in elucidating the structural organization of the 10-nm fiber and are currently being used to study the higher order 30-nm fiber [reviewed by Butler (1983)]. Furthermore, they have been used to detect and illustrate structural differences between transcriptionally inactive and transcriptionally active domains of chromatin (Weintraub & Groudine, 1976; Bloom & Anderson, 1978, 1979; Weisbrod, 1982). In addition, the "digestibility" of chromatin at various stages of the cell cycle has been examined (Prentice et al., 1985). However, in the latter study, reproducible results could only be obtained when the ionic composition of the isolation buffer was carefully controlled (Prentice & Gurley, 1983). Quite clearly, a prerequisite for all such studies is the development of techniques for isolating nuclei in which the native structure of chromatin is maintained.

Studies on the folding of the 10-nm polynucleosome chain into the 30-nm condensed fiber, and even higher order forms, have shown that monovalent and more particularly divalent cations have critical effects on fiber structure in vitro [see review by Butler (1983)]. With this information in mind, we have examined the effects of salt and divalent cations on chromatin integrity as measured by its sensitivity to exogenous nucleases. The data show that marked changes in chromatin structure can occur both during the isolation of nuclei and

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