Semisynthetic Derivatives of Glucagon: (Des-His^1)N^ϵ-acetimidoglucagon and N^α-Biotinyl-N^ϵ-acetimidoglucagon†

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ABSTRACT: N^ϵ-Acetimidoglucagon to be used for semisynthesis was prepared by reacting glucagon with methyl acetimidate hydrochloride at pH 10.2, favoring acetimidation of the sole ϵ-amino group. N^ϵ-Acetimidoglucagon was isolated from the crude acetimidoglucagon mixture by anion-exchange chromatography at pH 9.4, producing a derivative which was identical with native glucagon on isoelectric focusing and which by amino acid analysis had greater than 98% of the lysine blocked. The yield was greater than that obtained when tetrahydrophthalic anhydride was used as a chromatographic handle to remove peptides with unreacted amino groups. N^ϵ-Acetimidoglucagon closely resembled native glucagon in its biological activity and binding affinity, eliminating the need for deprotection. Semisynthetic N^α-biotinyl-N^ϵ-acetimidoglucagon, prepared by reacting (*N*-hydroxysuccinimido)biotin with N^ϵ-acetimidoglucagon and purified by cation-exchange chromatography, was homogeneous upon isoelectric focusing (p*I* = 5.2) and exhibited 1.2% of the binding affinity, 2.4% of the biological potency, and 30% of the maximum activity of the native hormone. Preliminary fluorescence microscopy demonstrated binding of N^α-biotinyl-N^ϵ-acetimidoglucagon to glucagon specific receptors following exposure to fluorescein-labeled avidin. Capping of labeled receptors could be visualized with time. (Des-His^1)N^ϵ-acetimidoglucagon, prepared via a manual Edman degradation of N^ϵ-acetimidoglucagon and isolated by cation-exchange chromatography, was homogeneous upon isoelectric focusing (p*I* = 5.2). The second residue, serine, has also been removed. Semisynthetic coupling of alternative residues to such derivatives will provide insight into the role of the amino-terminal residues in mediating the biological actions of the hormone.

The peptide hormone glucagon secreted by the α cells of the pancreatic islets has numerous well-known effects upon hepatocyte fuel metabolism (Watkins et al., 1977; Snodgrass et al., 1978; Schworer & Mortimore, 1979) as well as interesting and perhaps valuable effects upon other tissues such as heart (Glick et al., 1968; Parmley et al., 1969), kidney (Spark et al., 1975; Bailly et al., 1980), regenerating cells (Paul et al., 1972), and brain (Conlon et al., 1979; Tager et al., 1980). Furthermore, it is reported to be a growth factor for cultured adenocarcinoma cells (Murakami & Masui, 1980).

A number of studies have sought to evaluate the role of specific amino acids in the interaction of glucagon with its receptors. Modifications of the α- and ϵ-amino groups have included acetylation (Desbuquois, 1975; Epand et al., 1981), trinitrophenylation (Epand & Wheeler, 1975; Cote & Epand, 1979; Bregman et al., 1980; Epand et al., 1981), carbamoylation and guanidation (Bregman et al., 1980; Epand et al., 1981), and amidination (Wright & Rodbell, 1980). Preservation of the charge of the single lysine at position 12 appears essential for binding, while histidine-1 appears to be necessary for both binding and activation of adenylate cyclase. Whether perturbation of these functions resulting from the histidine modification is due to a direct effect of modifying the amino group or whether it is due to steric hindrance of the imidazole group or an alteration in its proton dissociation pattern is currently uncertain.

Little is known of the interactions of glucagon with other tissues. Most of the effects are generally considered to be mediated through cAMP-dependent mechanisms, although cAMP-independent systems are recognized as well (Cote & Epand, 1979; Khan et al., 1980). The fact that glucagon effects are produced in some tissues at elevated concentrations of hormone implies that the hormone–receptor interactions in these tissues may differ from those in hepatocyte membranes.

Since the diabetic individual frequently has an elevated concentration of circulating glucagon (Unger & Orci, 1981), it is important to characterize the tissues and cells which respond to glucagon. It is also necessary to identify more fully the mechanism of interaction with these receptors as well as hepatocyte receptors in order to comprehend fully the implications of the abnormal hormone concentrations. It may well be that elevated concentrations of glucagon bring about events in separate tissues which are poorly understood. Certainly the tight conservation of glucagon throughout evolution suggests that glucagon may have critical actions in addition to its counterregulation of insulin.

The semisynthetic preparation of N^α-biotinyl-N^ϵ-acetimidoglucagon described here was designed therefore for a number of reasons: (1) The preparation and isolation of N^ϵ-acetimidoglucagon in good yield and high purity make additional semisynthetic modification of the amino terminus an immediate and viable possiblity. (2) The binding affinity and biological activity of N^ϵ-acetimidoglucagon which is nearly the same as the native hormone confirm the need for charge preservation on the lysine residue and also suggest that further derivatives can be produced and examined without the need to deprotect the lysine. (3) Quantitative removal of histidine-1 by manual Edman degradation of N^ϵ-acetimidoglucagon can be accomplished and (des-His^1)N^ϵ-acetimidoglucagon can be easily isolated by cation-exchange chromatography to give a preparation to which may be added alternative or labeled residues. (4) The active ester coupling is an efficient method to use for the addition of desired groups to the amino terminus. (5) The specific addition of the biotinyl moiety extends the number of derivatives of the α-amino group which have di-

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minished binding and activity. (6) The preparation of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon provides a covalently modified derivative with which to probe the histologic localization of glucagon in a variety of tissues at the light or electron microscopic level by taking advantage of the high affinity of fluorescent or gold-labeled avidin for the biotinyl moiety (May et al., 1978; Bayer & Wilchek, 1980). Such a probe can be a valuable tool for the localization of receptors within tissues as well as for determining the fate of the hormone in specific cells following binding.

Experimental Procedures

Materials

Crystalline porcine glucagon (lot no. 258-D30-138-2) was provided through the courtesy of Eli Lilly and Co. IODO-GEN,[1] trifluoroacetic acid (Sequanal grade), PITC (Sequanal grade), and (*N*-hydroxysuccinimido)biotin were purchased from Pierce Chemical Co. "Ultrapure" grade Tris base was obtained from Schwarz/Mann, and dithiothreitol and APM came from Sigma Chemical Co. Carrier-free [^{125}I]iodine in NaOH (>350 mCi/mL), [^{3}H]cAMP (30–50 Ci/mmol), and Aquasol scintillation cocktail were purchased from New England Nuclear, and [α-^{32}P]ATP (10–25 Ci/mmol) was purchased from ICN. BSA and unlabeled cAMP and ATP came from Calbiochem, and GTP, creatine phosphate, and creatine phosphokinase were from Boehringer Mannheim Corp. Male Sprague-Dawley rats (140–160 g) were obtained from Laboratory Supply Co., Indianapolis, IN.

The Sephadex G-10 and G-25F gel filtration media were obtained from Pharmacia and the CM-cellulose (CM52) and DEAE-cellulose (DE52) ion-exchange resins from Whatman. Dowex AG 50W-X4 (200–400 mesh, H^+ form), Dowex AG 1-X8 (100–200 mesh, Cl^- form), and the materials required for isoelectric focusing came from Bio-Rad Laboratories. Woelm neutral alumina (activity grade 1) was obtained from ICN Nutritional Biochemicals. Oxoid "Nuflow" filters came from Medox Chemicals (Ottawa, Canada), and Spectrapor 6 dialysis tubing was from Cole-Parmer. Methyl acetimidate hydrochloride was prepared according to the method of Hunter & Ludwig (1962). All solutions of urea were freed of cyanate immediately before use by passage through a 2.5 × 50 cm column of Rexyn I-300 (Fisher Scientific Co.). *N*,*N*-Dimethylformamide was redistilled before use and was stored at 4 °C over a molecular sieve. All other chemicals and reagents were the best grade commercially available and were used without further purification. Distilled-deionized water was used throughout.

Methods

Purification of Native Glucagon. Glucagon was purified by cation-exchange chromatography on a CM-cellulose column (2.5 × 13 cm) equilibrated with a first buffer of 10 mM ammonium acetate in 6 M urea, pH 5.2. The peptide was eluted at 45 mL/h with a 22-h linear gradient formed between the first buffer and a second buffer of 50 mM ammonium acetate in 6 M urea, pH 5.2. Desalting prior to lyophilization and storage at −20 °C were as described (England et al., 1982).

Preparation and Purification of N^{ϵ}-Acetimidoglucagon. Purified glucagon (12 mg, 3.44 μmol) was dissolved in 5 mL of 0.1 M disodium tetraborate buffer, pH 10.2. The solution was immediately cooled to 15 °C, a 50-fold excess of methyl acetimidate hydrochloride (19 mg, 172 μmol) was added, and the solution was stirred at this temperature for 30 min while the pH was maintained at 10.2. The reaction was terminated by gel filtration on a Sephadex G-10 column (1.6 × 95 cm), the fractions were eluted with 0.2 M ammonium carbonate, and the peptide was lyophilized for storage. A DEAE-cellulose column (0.9 × 60 cm) was equilibrated at 4 °C with 10 mM Tris-HCl in 6 M urea, pH 9.4, the crude acetimidoglucagon was added, and the peptides were eluted at 12 mL/h with a 40-h linear gradient formed from equal volumes of the first buffer and a second buffer of 200 mM Tris-HCl in 6 M urea, pH 9.4. The major peptide fraction was collected and desalted on a Sephadex G-25 column (3 × 100 cm) eluted with 5% acetic acid. The peptide fraction was lyophilized and stored at −20 °C.

Characterization of N^{ϵ}-Acetimidoglucagon by Reaction with Phenyl Isothiocyanate. N^{ϵ}-Acetimidoglucagon was reacted with PITC by using a procedure similar to that reported by Bregman & Hruby (1979). The peptide (3 mg, 0.8 μmol) was dissolved in 0.75 mL of 67% aqueous pyridine. A 7.4% solution of PITC in pyridine (0.32 mL) was added to the peptide solution. The mixture was incubated in a nitrogen atmosphere at 37 °C for 1 h. Excess PITC was extracted with benzene, and the aqueous layer was dried in vacuo. Dithiothreitol (3 mg) was added to the dried sample along with the trifluoroacetic acid (1.2 mL) to prevent oxidation of the peptide. After 30 min in the dark at room temperature, the trifluoroacetic acid was evaporated. The sample was then dissolved in 5% acetic acid and desalted as described above. After lyophilization, (des-His^1)N^{ϵ}-acetimidoglucagon was isolated by cation-exchange chromatography on a CM-cellulose column (1.5 × 15 cm) equilibrated with a first buffer of 10 mM ammonium acetate in 6 M urea, pH 4.5. The peptides were eluted at 30 mL/h with a 24-h linear gradient formed between the first buffer and a second buffer of 100 mM ammonium acetate in 6 M urea, pH 5.0. The peptide fraction was desalted on a Sephadex G-25 column (3 × 100 cm) with 5% acetic acid.

End-Group Analysis of (Des-His^1)N^{ϵ}-acetimidoglucagon. (Des-His^1)N^{ϵ}-acetimidoglucagon (0.35 mg, 0.1 μmol) was reacted with PITC as described above. Following benzene extraction, the aqueous layer was dried. The sample was then incubated with trifluoroacetic acid for 30 min. After the trifluoroacetic acid was evaporated, the residue was dissolved in water (0.1 mL), and the thiazolinone amino acids were extracted 3 times with ethyl acetate (0.2 mL). The solvent was evaporated under a nitrogen stream prior to converting the thiazolinone amino acids to the corresponding phenylthiohydantoin derivatives according to Edman & Henschen (1975). The residue containing the thiazolinone amino acids was dissolved in 0.2 mL of 1 N HCl containing 1 μL/mL ethanethiol. The tube was filled with nitrogen, sealed, vortexed, and heated at 80 °C for 10 min. After the sample was cooled to room temperature, the PTH-amino acids were extracted twice with ethyl acetate (0.7 mL) which was then evaporated under nitrogen. The sample was stored at −20 °C until further analysis.

Identification of PTH Derivatives of Amino Acids by HPLC. A procedure similar to one described by Harris et al. (1980) was used to separate PTH-amino acids by HPLC on a Varian MicroPak MCH-5 ODS column (5-μm particle size; 0.4 × 30 cm). Samples (5 nmol) were loaded in 10 μL of

[1] Abbreviations: IODO-GEN, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; PITC, phenyl isothiocyanate; APM, leucine aminopeptidase (microsomal); BSA, bovine serum albumin; EDTA, disodium ethylenediaminetetraacetate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; THPA, 3,4,5,6-tetrahydrophthalic anhydride; Tris, tris(hydroxymethyl)aminomethene; other abbreviations are those recommended by IUPAC–IUB.

acetonitrile–90% formic acid (8:2). The PTH-amino acids were eluted at 42 mL/h with a 30-min linear gradient formed by solutions of 10 mM sodium acetate (pH 3.7) and acetonitrile, with the first solution being in the ratio of 9:1 and the final solution in the ratio 1:9. The column effluent was monitored for absorbance at 269 nm. Standards of PTH-derivatives of serine, glutamine, glutamic acid, and histidine were run for comparison.

Amino Acid Analyses. Routine acid hydrolysates were prepared in 6 N HCl at 110 °C for 24 h. Since the acetimidyl group is partially removed during acid hydrolysis, two procedures were used to correct for this destruction in the analysis of N^{ϵ}-acetimidoglucagon. In preliminary studies peptide samples were hydrolyzed for 24, 48, or 72 h. The amount of lysine present at each time was quantitated, allowing the amount of acetimidolysine present to be determined by extrapolation to zero time. In the preparation reported here, the quantity of unreacted lysine was determined by an enzymatic digest with APM. The digest was performed at 37 °C for 24 h in 0.05 M phosphate buffer (pH 7.8) containing 0.5 mg/mL chloramphenicol. A digest blank was also prepared and analyzed. The analyses were performed on a Beckman 120 C amino acid analyzer either equipped with a DC-6A resin (Durrum) or fitted with a column (0.9 × 19 cm) of PA-35 resin (Jones et al., 1978).

Preparation and Purification of N^{α}-Biotinyl-N^{ϵ}-acetimidoglucagon. The biotinylation procedure used was a modification of that described by Bayer & Wilchek (1980). Purified N^{ϵ}-acetimidoglucagon (5.5 mg, 1.5 μmol) was dissolved in 0.5 mL of reaction buffer which consisted of DMF–0.01 N HCl–50 mM KH_2PO_4 (pH 7.0) (1.7:1.3:1). The apparent pH of the reaction buffer was 8.2. A 25-fold molar excess of (*N*-hydroxysuccinimido)biotin (13.2 mg, 38.6 μmol) in 6.4 mL of reaction buffer was added to the peptide solution. The reaction was terminated after 4 h at 20 °C by lowering the pH to approximately 3 with glacial acetic acid followed by desalting on a Sephadex G-25 column (3 × 100 cm) eluted with 5% acetic acid. The reaction products were separated by cation-exchange chromatography on a CM-cellulose column (1.5 × 15 cm) equilibrated with 10 mM ammonium acetate in 6 M urea, pH 4.5. The peptides were eluted at 30 mL/h with a 16-h linear gradient formed from equal volumes of first buffer and a second buffer of 70 mM ammonium acetate in 6 M urea, pH 5.2. The peptide was desalted as described above, lyophilized, and stored at −20 °C.

Isoelectric Focusing. Isoelectric focusing was carried out on 7.5% polyacrylamide tube gels (5 × 110 mm) containing 6 M urea and 2% (w/v) pH 3–10 carrier ampholytes as described previously (England et al., 1982). The sensitivity of Coomassie Blue G staining determined by focusing native glucagon at concentrations from 1 to 50 μg yielded a detection limit of 2 μg and was superior to that obtained with silver staining.

Biological Assays. Binding and adenylate cyclase assays were performed as described by England et al. (1982), except that the incubation buffer used for the glucagon binding assay was 30 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.1% BSA. Results of the adenylate cyclase assays are expressed as percent of activation over basal level which averaged about 0.4 nmol of cAMP formed per mg of membrane protein in 10 min. Maximum activity was 3 to 4 times the basal level activity. In the binding assays nonspecific binding measured in the presence of excess unlabeled peptide was 5–10% of total binding and was subtracted from the total to give specific binding. Results are expressed as the percent of maximum specific binding which was about 1 × 10^5 cpm/mg of membrane protein.

Table I: Amino Acid Composition of N^{ϵ}-Acetimidoglucagon, (Des-His[1])N^{ϵ}-acetimidoglucagon, and N^{α}-Biotinyl-N^{ϵ}-acetimidoglucagon

amino acid	enzymatic hydrolysis: N^{ϵ}-acetimidoglucagon	enzymatic hydrolysis: (des-His[1])-N^{ϵ}-acetimidoglucagon	N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon (acid hydrolysis)
Asp	2.84 (3)[a]	3.29 (3)	4.00 (4)
Thr	2.93 (3)	2.98 (3)	2.85 (3)
Ser	3.87 (4)	3.94 (4)	3.06 (4)
Glu	0.00 (0)	0.00 (0)	2.98 (3)
Gly	0.83 (1)	0.87 (1)	1.05 (1)
Ala	1.18 (1)	1.11 (1)	0.95 (1)
Val	1.11 (1)	0.85 (1)	0.96 (1)
Met	1.00 (1)	0.76 (1)	1.03 (1)
Leu	1.90 (2)	2.03 (2)	2.07 (2)
Tyr	1.80 (2)	1.88 (2)	1.93 (2)
Phe	2.05 (2)	1.88 (2)	1.91 (2)
Lys	0.02[b] (0)	0.06[b] (0)	0.52[b] (1)
His	0.85[b] (1)	0.01[b] (0)	1.07[b] (1)
Arg	2.07[b] (2)	1.98[b] (2)	1.93[b] (2)
Trp	1.11[b] (1)	1.02[b] (1)	ND[c]

[a] Expected values in parentheses are based on the glucagon sequence. [b] These values were obtained from analysis on the PA-35 column. [c] ND, not determined.

Results

Reaction Scheme. The reaction scheme utilized for this study is shown in Figure 1. The pH of 10.2 favors the acetimidation of ε-amino groups over α-amino groups (Hunter & Ludwig, 1962; DiMarchi et al., 1978). At this temperature and pH the loss of methyl acetimidate to hydrolysis should be minimized (Browne & Kent, 1975; DiMarchi et al., 1978), allowing for a more complete reaction. Performing the anion-exchange chromatography at pH 9.4 takes advantage of the p*K* difference between the acetimidyl group and the ε-amino group of lysine, allowing the possible acetimidoglucagon derivatives to be separated. This isolation procedure proved superior to that employing tetrahydrophthalic anhydride as a chromatographic handle to add a negative charge to all unreacted amino groups in the sense that a higher yield of homogeneous product could be obtained. Both preparations, however, gave essentially the same biological activity (below).

In preliminary studies over a wide range of pH values, selective biotinylation of the α-amino group of native glucagon was not obtained, and even at pH 5–6 where α-biotinylation should have been favored, a substantial proportion of the ε-amino groups was biotinylated. This observation is in contrast to the report (Bayer & Wilchek, 1980) that the reaction with (*N*-hydroxysuccinimido)biotin could be restricted mostly to α-amines of many proteins at this pH value.

Purification and Characterization of N^{ϵ}-Acetimidoglucagon. The elution profile for the purification of crude acetimidoglucagon is shown in Figure 2. The major fraction (peak III) was identified as N^{ϵ}-acetimidoglucagon by amino acid analysis as shown in Table I. Inasmuch as acetimidolysine is partially converted to lysine during acid hydrolysis, an enzymatic hydrolysis was used to quantitate the amount present. Because of poor resolution of acetimidolysine from histidine as well as lysine from ammonia on the DC-6A column, the PA-35 column was required to optimize the quantitation of these two amino acids. After the digest blank was corrected, 2% lysine was detected in the sample, indicating that it contained approximately 98% acetimidolysine. Acetimidolysine was noted to be present in large quantity in the eluate of the DC-6A

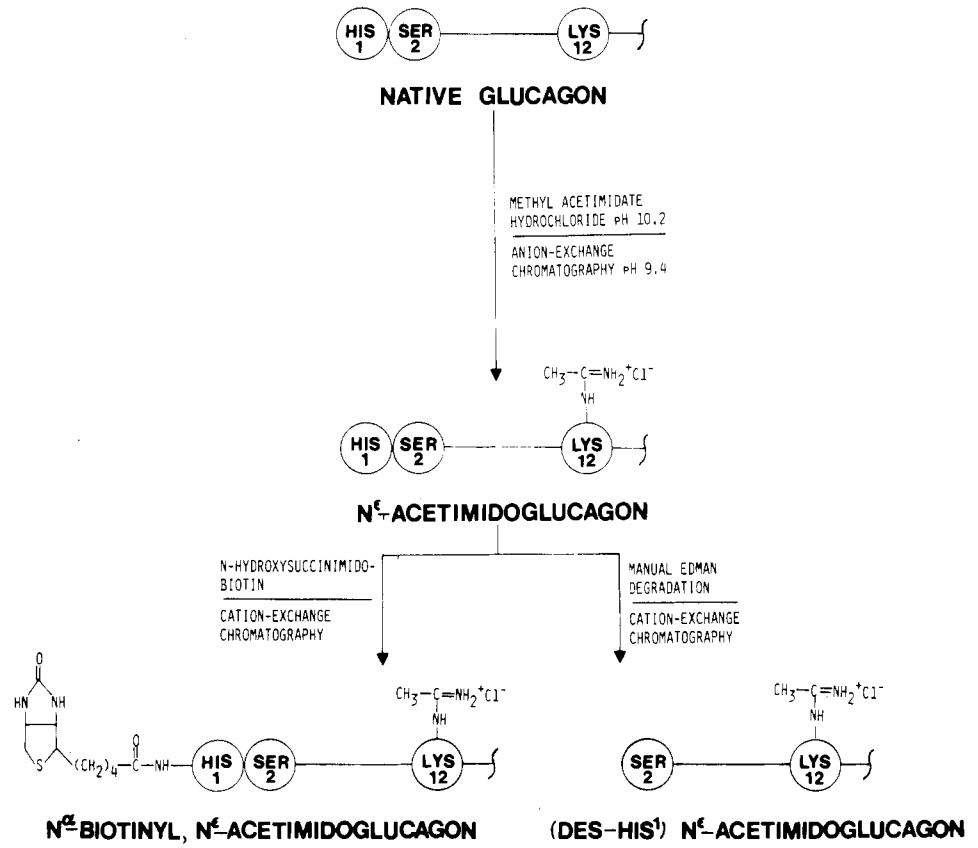

FIGURE 1: Summary of the reaction scheme employed to generate N^{ϵ}-acetimidoglucagon, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon, and (des-His^1)N^{ϵ}-acetimidoglucagon.

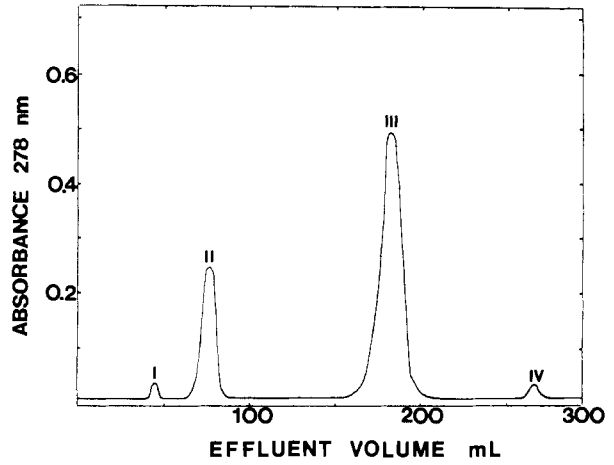

FIGURE 2: DEAE-cellulose anion-exchange chromatography of the glucagon derivatives generated by the acetimidation procedures. Details are given in the text.

column, but because of its overlap with histidine, it was necessary to quantitate acetimidolysine indirectly on the basis of the loss of free lysine as described above. The N^{ϵ}-acetimidoglucagon was found to focus identically to native glucagon (pI = 6.5). When the N^{ϵ}-acetimidoglucagon was subjected to a manual Edman degradation as described in the following section and the products were separated by cation-exchange chromatography, greater than 95% of the material was converted to (des-His^1)N^{ϵ}-acetimidoglucagon, indicating that the α-amino group was not acetimidated. The maximum contamination with native glucagon (~2%) should not interfere with biological characterizations of the subsequent biotinyl derivatives prepared from N^{ϵ}-acetimidoglucagon since the reactivity of the unprotected ϵ-amino group favors biotinylation at this group removing a charge. The chromatographic purification procedures used in preparation of subsequent derivatives would eliminate essentially all the dibiotinylglucagon derived from native hormone.

Comparison of the peak areas in Figure 2 indicates that N^{ϵ}-acetimidoglucagon, peak III, accounts for 75% or more of the possible acetimido derivatives. On the basis of the expected charge differences of the possible acetimidoglucagon derivatives at pH 9.4, peaks I and II are thought to be derivatives in which the α-amino group is acetimidated, and peak IV is unreacted native glucagon. Further characterization of these additional peaks was not attempted.

Purification and Characterization of (*Des-His*^{1})N^{ϵ}*-acetimidoglucagon.* A manual Edman degradation of N^{ϵ}-acetimidoglucagon followed by cation-exchange chromatography gave approximately a 95% yield of (des-His^1)N^{ϵ}-acetimidoglucagon based on an estimate of peak areas. On the basis of their elution positions, the minor contaminants separated during the purification are likely to be unreacted N^{ϵ}-acetimidoglucagon or N^{α},N^{ϵ}-diacetimidoglucagon and a derivative in which the small amount of unprotected lysine has reacted with the PITC. (Des-His^1)N^{ϵ}-acetimidoglucagon was homogeneous upon isoelectric focusing with a pI of 5.2.

Conflicting reports exist regarding the lability of an amino-terminal histidine during the Edman degradation. In some reports both histidine and the amino acid following it in the sequence were released as PTH derivatives (Blombäck et al., 1967; Schroeder, 1967), while this was not observed by Edman & Henschen (1975). Although this phenomenon is not completely understood, the coupling buffer seems to have an effect (Thomsen et al., 1972; Edman & Henschen, 1975), and the abnormal degradation can be minimized by an extraction with benzene immediately at the end of the coupling stage, before the drying takes place (Thomsen et al., 1972). A second

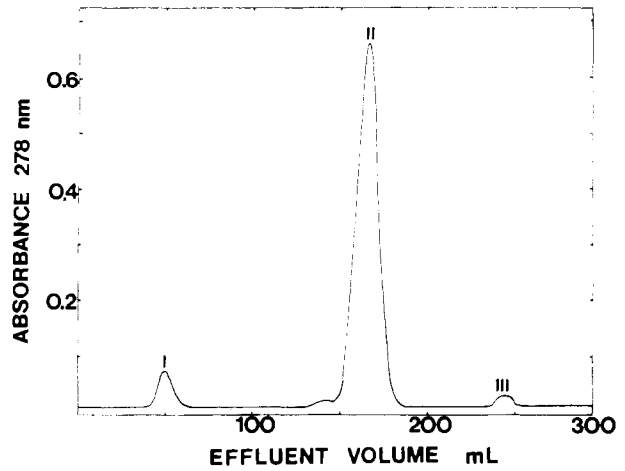

FIGURE 3: CM-cellulose cation-exchange chromatography of crude N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon. Details are given in the text.

Edman degradation was performed on (des-His[1])N^{ϵ}-acetimidoglucagon to determine if this double cleavage had occurred during our Edman degradation. The thiazolinone amino acids were extracted and converted to the corresponding PTH derivatives. Unfortunately, the recovery of PTH-serine is not quantitative because it undergoes a β-elimination reaction followed by other reactions, presumably polymerization. The conversion conditions used here minimize the tendency for β elimination and allow PTH-serine to be obtained in 40% yield (Edman & Henschen, 1975). Analysis of the PTH-amino acids by HPLC showed a major peak of PTH-serine (11.7 mL) and minor peaks of PTH-glutamine (12.5 mL) and PTH-glutamic acid (13.1 mL) (data not shown). An unidentified minor peak eluted at 13.4 mL. Even if no adjustment were made for the low recovery of PTH-serine, it comprises 88% of the PTH-amino acids, as judged by integration of the peaks. These preliminary results indicate that a second manual Edman degradation can be used to prepare (des-His[1],Ser[2])N^{ϵ}-acetimidoglucagon. The amino acid analysis shown in Table I confirms the absence of histidine.

Purification and Characterization of N^{α}-Biotinyl-N^{ϵ}-acetimidoglucagon. Following the reaction of the N^{ϵ}-acetimidoglucagon with (*N*-hydroxysuccinimido)biotin, separation of the reaction products by cation-exchange chromatography resulted in the elution profile illustrated by Figure 3. One major and two minor peaks were well separated, and material from peak II was homogeneous on isoelectric focusing with a p*I* of 5.2. Isoelectric focusing performed on a 200-μg aliquot taken from peak II showed no evidence of a band migrating to the position of native glucagon or N^{ϵ}-acetimidoglucagon. Since the detection limit was determined to be approximately 2 μg, the maximum contamination with either derivative is therefore approximately 1%. The amino acid analysis after acid hydrolysis of this derivative is shown in Table I. A full residue of histidine is present since the amide bond between the biotin and the α-amino group is cleaved during hydrolysis. Lysine is present in the acid hydrolysis as expected since acetimidolysine is unstable in the acid digest used. However, acetimidolysine would be expected to remain essentially intact during the biotinylation reaction at pH 8.2 for the limited time of the reaction since the half-life for deprotection in concentrated ammonium hydroxide–acetic acid (15:1 v/v) at pH 11.5 is approximately 4 h (DiMarchi et al., 1978).

Greater than 90% of the possible biotinylglucagon derivatives is N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon as judged by the peak areas in Figure 3. Peak III is presumed to be unreacted N^{ϵ}-acetimidoglucagon, since native glucagon elutes from this column in the same position. Peak I is believed to be N^{α},N^{ϵ}-dibiotinylglucagon, since this derivative would elute from a cation-exchange column very rapidly due to the fact that both the positive charges of the α- and ϵ-amino groups are blocked. A small amount of this derivative is not surprising, since the analysis of the N^{ϵ}-acetimidoglucagon does not preclude contamination with $\sim$2% of glucagon unprotected at lysine-12. In a number of preliminary studies unprotected ϵ-amino groups were found to be extremely reactive with (*N*-hydroxysuccinimido)biotin at pH 8.2 as suggested by previous studies with insulin (Hofmann et al., 1977; May et al., 1978).

Biological Characterization of Glucagon Derivatives. In agreement with the results of Wright & Rodbell (1980), both preparations of N^{ϵ}-acetimidoglucagon were found to resemble native glucagon closely in their ability to activate adenylate cyclase and to bind to rat liver plasma membranes as shown in parts A and B, respectively, of Figure 4. The concentration of peptide giving half-maximal activation was 2.7×10^{-9} M for native glucagon and 6.9×10^{-9} M for N^{ϵ}-acetimidoglucagon, while the peptide concentration displacing 50% of mono[^{125}I]iodoglucagon from membranes was 3.2×10^{-9} M for native glucagon and 5.4×10^{-9} M for N^{ϵ}-acetimidoglucagon. Preparation of N^{ϵ}-acetimidoglucagon making use of a chromatographic handle for separation gave a concentration for half-maximum activation of 5.7×10^{-9} M. All preparations showed the derivative to be a full agonist.

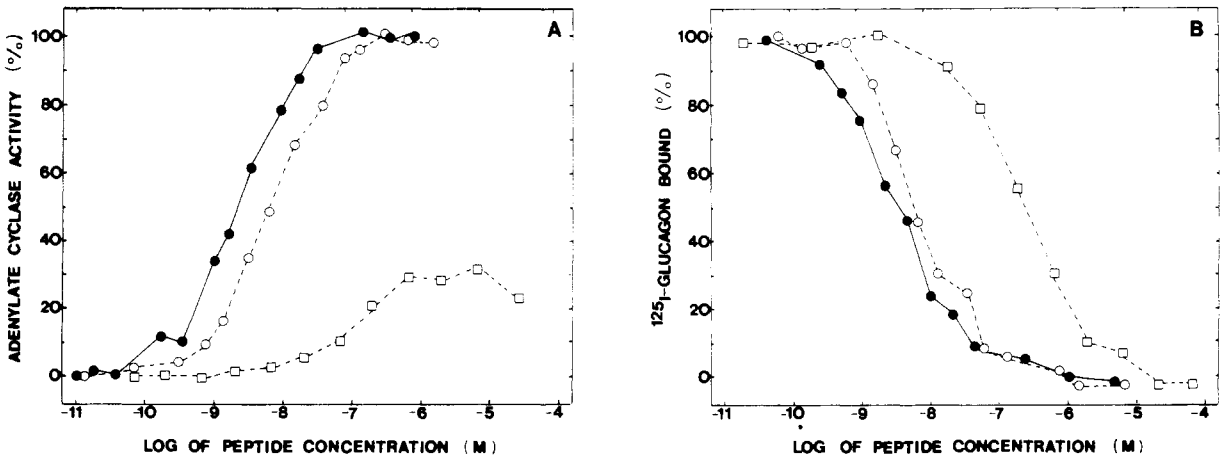

FIGURE 4: Biological characterization of derivatives. (A) Dose–response curves for the activation of rat liver adenylate cyclase. (●) Native glucagon; (○) N^{ϵ}-acetimidoglucagon; (□) N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon. (B) Displacement of mono[^{125}I]iodoglucagon bound to rat liver plasma membranes. (●) Native glucagon; (○) N^{ϵ}-acetimidoglucagon; (□) N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon.

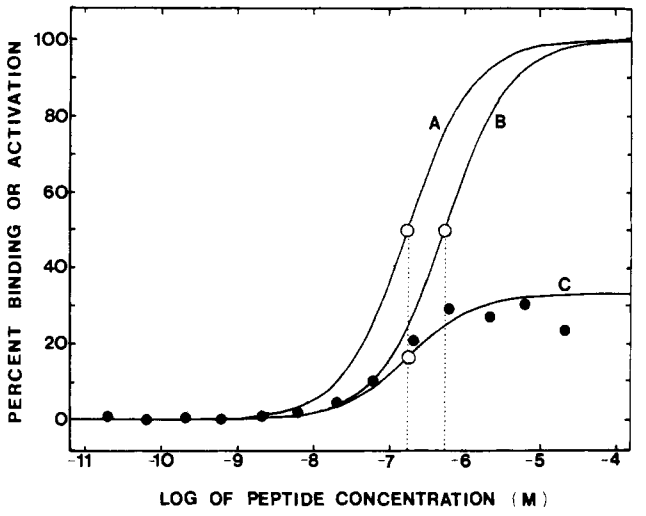

FIGURE 5: Theoretical binding and activation curves for an antagonist contaminated with an agonist. (Curve A) Equilibrium binding curve for the mixture of agonist and antagonist. (Curve B) Equilibrium binding curve and activation curve for the agonist in the absence of competitive binding by the antagonist. (Curve C) Agonist binding in the presence of competitive binding by the antagonist. This curve also represents the activation for the mixture of agonist and antagonist. (●) Experimental data for the activation of adenylate cyclase by N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon. (○) Curve midpoint. Curves were calculated with the antagonist $K_D = 2.7 \times 10^{-7}$ M, the agonist $K_D = 5.9 \times 10^{-9}$ M, and the agonist concentration equal to 1% of the total peptide.

Parts A and B of Figure 4 also show the activation and binding of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon. The concentration of this derivative displacing 50% of mono[^{125}I]iodoglucagon from membranes was 2.7×10^{-7} M, giving a relative binding affinity of 1.2%, while the concentration required for half-maximal activation was 1.1×10^{-7} M, giving a relative biological potency of 2.4% (England et al., 1982). As can be noted from Figure 4 the activation curve for N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon does not attain 100% of the activity of native glucagon. This could be due to its partial agonist character or due to low level contamination of an antagonist derivative with fully active hormone.

Figure 5 represents the theoretical binding and activation curves of an antagonist contaminated with a trace amount of a high-affinity agonist. Percent binding of the agonist and antagonist can be calculated from (Ross et al., 1977)

$$P_H = \frac{[H]/K_H}{1 + [H]/K_H + [I]/K_I} \times 100 \quad (1)$$

$$P_I = \frac{[I]/K_I}{1 + [H]/K_H + [I]/K_I} \times 100 \quad (2)$$

where H and I refer to the agonist and antagonist species, respectively. Dissociation constants for the agonist (K_H) and antagonist (K_I) were taken to be those of N^{ϵ}-acetimidoglucagon and N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon, respectively, and the agonist concentration was taken to be 1% of the total peptide concentration. Experimental results of the activation assay for the N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon derivative are superimposed.

Curve A in Figure 5 represents the equilibrium binding curve for the mixture of agonist and antagonist. The half-maximal binding is displaced from that of native glucagon as a result of the binding contribution of the antagonist present at much higher concentration. However, the 1% agonist contaminant does contribute about one-third of the total binding, because of its much higher affinity.

If the antagonist did not effectively compete for binding sites with the 1% high-affinity agonist, then the binding would be described by curve B (Figure 5). In this case the activation curve would be identical with the binding curve, and maximal (100%) binding would result in maximal activation. The apparent K_d for this curve would be exactly 100 times the K_d of N^{ϵ}-acetimidoglucagon or native glucagon since the agonist represents 1% of the total peptide.

Curve C of Figure 5 shows the agonist binding that contributes to the total binding (curve A) of the agonist and antagonist mixture. Thus curve C also represents the activation curve for the agonist and antagonist mixture. Partial agonism is observed because competition for binding sites prevents full binding site occupancy by the agonist even at saturating concentrations. The concentration for half-maximal activation (curve C) is the same as that for half-maximal binding (curve A) because, even though activation reflects only the agonist binding, both activation and binding are controlled by the same overall binding equilibrium.

We have not determined in this report that the chromatographically homogeneous fraction of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon is completely free of low-level contamination with active hormone. If the derivative is completely pure, then the adenylate cyclase assay does in fact demonstrate its partial agonist characteristics. However, it is clear from the above discussion that our results are also completely consistent, with N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon being an effective antagonist whose apparent activity is due to trace contamination with an active hormone.

Direct demonstration of the binding capability of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon has been accomplished by the microscopic visualization of binding of the derivative to glucagon-specific receptors. Cultured Chinese hamster ovary cells were incubated for 10 min in the presence of 3×10^{-6} M N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon in Hank's balanced saline buffer and then washed. At 37 °C, in the presence of fluorescein-labeled avidin, diffuse binding was followed by capping. At 0 °C, no capping occurred. Prior incubation of cells with native glucagon at an equivalent concentration blocked the binding and capping phenomenon.

Discussion

This report details a semisynthetic procedure of value for understanding the relationship of hormone structure to its binding to receptors and activation of adenylate cyclase, as well as for exploring the cellular and subcellular localization of glucagon receptor sites in a variety of tissues. The protocol developed depends first upon the protection of the highly reactive and nucleophilic ϵ-amino group of the sole lysine with the acetimidyl group. This protecting group has been widely used in chemical modification studies and for protein semisynthesis. In many cases the fully acetimidated protein retains its biological activity (Slotboom & de Haas, 1975; Harris & Offord, 1977; Fölsch, 1978) or exhibits physical properties essentially identical with those of the unmodified protein (Harris, 1977; DiMarchi et al., 1978), and deprotection of the modified protein may not be necessary. This conservation of function may be attributed to the conservation of the charge on the lysine by the acetimidyl group allowing the maintenance of electrostatic interactions which stabilize higher structural integrity. Preservation of the charge of lysine-12 of glucagon also permits essentially full expression of the binding and activation of glucagon.

The acetimidation procedure reported here differs from that reported by Wright & Rodbell (1980) in that less methyl

acetimidate, shorter reaction time, and lower temperatures were used. These milder conditions may in part account for the higher yield of a more homogeneous product which we have obtained. Furthermore, our purification at pH 9.4 takes advantage of the difference of the pK values of the acetimidyl group and the lysine ϵ-amino group as opposed to the previously reported separation at pH 3 where no charge differences would allow separation.

Separation of the N^{ϵ}-acetimido hormone by anion-exchange chromatography at pH 9.4 provides a product which is identical with native glucagon upon isoelectric focusing. Evidence indicates that in this product the lysine is fully protected, while the α-amino group is free. N^{ϵ}-Acetimidoglucagon was shown to be a full agonist. Binding to rat liver plasma membranes and activation of adenylate cyclase in these membranes were shown to be comparable to that of native glucagon. The product obtained by this procedure is similar in amino acid composition, pI, binding, and adenylate cyclase activity to that obtained by the more cumbersome use of the chromatographic handle, THPA, and its subsequent removal (Mar et al., 1981), but the yield was improved.

Protection of the lysine residue of glucagon with the acetimidyl group was found to be necessary before the hormone could be biotinylated by coupling with the active ester, (*N*-hydroxysuccinimido)biotin. This protection avoids biotinylation of the lysine whose charge is necessary for the binding process (Epand, 1980). However, the acetimidyl group appears to be stable to the subsequent conditions used for the active ester coupling, and the reaction proceeds rapidly and fully. That the major reaction product is N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon is shown by the findings that the product was homogeneous by isoelectric focusing, the product had a pI indicating the loss of the amino-terminal charge, and amino acid analysis yielded 50% acetimidolysine even after 24-h acid hydrolysis. Also, the elution from a cation-exchange column was consistent with that of a glucagon derivative with a blocked α-amino group.

When tested for biological activity, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon, in contrast to N^{ϵ}-acetimidoglucagon, is apparently a partial agonist with decreased activity roughly comparable to the diminished binding. Studies in other laboratories characterizing derivatives with the α-amino group modified by the addition of an acetyl group (Desbuquois, 1975), a trinitrophenyl group (Epand et al., 1981), or a carbamoyl group (Epand et al., 1981) have suggested that the amino-terminal histidine plays a more important role for activation of adenylate cyclase than for binding, although binding is somewhat perturbed by its modification or loss (Lin et al., 1975).

Contamination of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon with less than 2% fully active hormone has not been completely ruled out. The apparent partial agonist activity could be a characteristic of the derivative itself or could result from low level contamination. However, the derivative has been shown to bind to glucagon-specific receptors on cells in culture, and the receptors were shown subsequently to cap. Therefore for the purpose of studying the characteristics of glucagon receptor binding and the subsequent fate of hormone and receptor, the derivative appears to be eminently suitable.

The addition of the biotinyl group to the acetimidated hormone is unlikely to cause a change in function significantly different from the addition of biotin to the native hormone unless some rather subtle change in the secondary structure of the hormone has occurred. Glucagon is known to be flexible and monomeric at low concentrations (Gratzer & Beaven, 1969). Preliminary studies of the circular dichroism spectra of N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon at low concentration have not revealed any marked change in secondary structure at pH 10.2.

Although detailed studies were not attempted, N^{α}-biotinyl-N^{ϵ}-acetimidoglucagon appears to be less soluble than native glucagon at pH 10.2 and at neutral pH. This feature might be attributed to the derivatization of the polar charged α-amino group with the biotinyl moiety which contributes four methylene groups in addition to the heterocyclic ring system. This hydrophobic enhancement may explain in part the fact that the biotinyl derivative is slightly less competent for binding than the more polar derivatives such as N^{α}-acetylglucagon which is reported to have only an 8-fold loss in binding compared to the 80-fold loss reported here. However, other influences of the biotinyl group cannot be evaluated on the basis of the data available.

A bulky polar disaccharide derivative of the α-amino group prepared in this laboratory (S. A. Coolican, unpublished results) appears to bind almost equally with the biotinylated derivative, yet be a nearly full agonist, suggesting that steric hindrance may not be as important as the change in chemical reactivity brought about by the hydrophobic character of the modified amino terminus. Such alterations in polar and electrostatic characteristics have been shown to have wide-ranging effects in other protein–protein interactions (Flanagan et al., 1981; Friend et al., 1981).

The choice of preparing the derivative of glucagon modified by the inclusion of a biotinyl group on the α-amino terminus rests upon the value of the preparation for three considerations. First, the strategy used for the preparation of the derivative requires the same protection of the highly reactive lysine amino group as would be required for replacing the amino-terminal histidine with alternative or labeled residues. The benign nature of the reaction conditions is demonstrated by the nearly full activity of the resulting N^{ϵ}-acetimidoglucagon derivative, and the (*N*-hydroxysuccinimido)biotin active ester coupling is efficient. In addition, the amino-terminal histidine has been removed and the des-His^{1} derivative isolated and characterized. Coupling of other residues, including regeneration of native glucagon by recoupling of L-histidine, is currently in progress and appears to proceed as anticipated (K. C. Flanders, unpublished results). The preparation of (des-His^{1},Ser^{2})N^{ϵ}-acetimidoglucagon will also allow replacement of serine-2.

Second, the biotinyl group modifies the amino-terminal histidine which, from previous studies, is known to be involved in activation of adenylate cyclase (Lin et al., 1975) through some transductive process still far from clear. These results extend those studies of other derivatives and confirm the importance of the amino terminus for both binding and activation.

Third, and of great biological interest, is the preparation of a derivative containing a covalently bound biotinyl group, which through its high affinity for avidin labeled with fluorescent tags or colloidal gold (Bayer & Wilchek, 1980) can be used as a morphologic marker for target cells containing receptors in a wide variety of tissues. Studies with antiglucagon antibody in conjunction with gold-labeled protein A have failed to reveal hepatocyte glucagon-specific receptors, even though the same technique demonstrates glucagon molecules stored in pancreatic A cells with high specificity (Floyd et al., 1981), suggesting that the necessary conformation for antiglucagon antibody binding may not be available in sufficient concentration in the tightly bound hormone–receptor complex.

The biotinylated hormone prepared in this report should be suitable for additional histologic studies. Evidence for binding

to membrane fragments has been presented, and binding to cultured cells has been visualized. That the binding is specific to glucagon receptors is indicated by appropriate controls in the presence of native glucagon. Additional studies aimed at defining the mechanisms involved in further metabolism and fate of both hormone and receptor are now possible and should contribute significantly to our understanding of the physiological effects of glucagon.

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