Semisynthetic Derivatives of Glucagon

THE CONTRIBUTION OF HISTIDINE-1 TO HORMONE CONFORMATION AND ACTIVITY*

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Semisynthetic N'-acetimidoglucagon was prepared from the [des-His']analogue by coupling the N-hydroxyysuccinimide ester of N'-tBoc-N''-imidazole-DNP-L-histidine to the peptide in dimethylformamide in the presence of 1-hydroxybenzotriazole. The deprotected, purified product was chemically identical to N'-acetimidoglucagon and equipotent to N'-acetimidoglucagon and native glucagon in its ability to activate adenylate cyclase and displace [125I]iodoglucagon from rat liver plasma membranes. Semisynthetic [Phe']-, [Ala']-, and [des-His']glucagon prepared similarly achieved 85, 55, and 35% of the maximal activity and 22, 2, and 6% of the binding potency of N'-acetimidoglucagon. The biological assays indicate that the amino group is involved to a greater extent in transduction than in binding, but the aromatic nature and hydrogen bonding capability of the imidazole ring of histidine-1 are important for both binding and transduction. In circular dichroism studies, all derivatives exhibited increased helicity in 2-chloroethanol. The [Phe'] analogue although less soluble behaved similarly to native glucagon, while the [Ala'] and [des-His']derivatives exhibited an increased helical content in 0.01 N HCl as a result of an increased propensity of these derivatives to self-associate in the absence of 2-chloroethanol. The unexpected conformational changes throughout the molecule may have relevance for the functional activity.

EXPERIMENTAL PROCEDURES

Materials

Crystalline porcine glucagon (lot No. 258-25J-215) was provided through the courtesy of Eli Lilly and Co. Trifluoroacetic acid (Sequant grade), N,N-dicyclohexylcarbodiimide, and 1-hydroxybenzotriazole monohydrate were purchased from Pierce Chemical Co. Dithiothreitol, N-ethylmorpholine, Trizma base, leucine aminopeptidase (microsomal), L-α-amino acid oxidase (Type V), 2-hydroxy-5-nitrobenzyl bromide, and the Nu-tBoc, N-hydroxysuccinimide ester of N'-acetimidoglucagon regenerated by semisynthesis to be identical to that prepared by acetimidation of native glucagon as well as to native glucagon itself. Histidine-1 was replaced with alanine lacking the imidazole ring system and with phenylalanine which possesses a hydrophobic aromatic ring. Secondary structure differences between the semisynthetic hormones are revealed by circular dichroic spectra. Functional studies show that the amino group and the imidazole ring system have separate roles, but each is involved in both binding and transduction.

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The abbreviations used are: N'-tBoc-N''-imidazole-DNP-histidine; ONSu, N-hydroxyysuccinimide ester; Trizma, 2-αmino-2-hydroxyethyl-1,3-propanediol.
of 50 mM ammonium acetate in 6 M urea, pH 5.2. A flocculent white precipitate was observed in the fractions corresponding to the native glucagon peak and direct application of the suspension to a gel filtration column revealed the urea resulted in poor recovery of peptide as also has been reported by Carrey and Epaud (1985). The fractions corresponding to the native glucagon peak were pooled and ammonium hydroxide (0.2 M) was added to 1 M until the precipitate was completely solubilized. It was then desalted on a Sephadex G-25F column (2.6 x 100 cm) eluted with 5% acetic acid. The peptide fraction was lyophilized and stored at -20°C.

Preparation and Purification of N'-Acetimidoglucagon—The acetimidation of native glucagon and the anion-exchange purification step were performed as previously described (Flanders et al., 1982). Since amino acid analysis of the material recovered from the anion-exchange column revealed low levels of histidine, the sample was further subjected to cation-exchange chromatography following the procedure described here for the purification of native glucagon to remove an impurity in which the α-amino group is blocked. The peptide-containing fractions from this column contained no precipitants making the addition of ammonium hydroxide before desalting unnecessary.

Preparation of [des-His']N'-Acetimidoglucagon—[des-His']N'- Acetimidoglucagon was prepared via an Edman degradation of N'-acetimidoglucagon according to Offord (1976). Cation-exchange purification was omitted unless amino acid analysis or isoelectric focusing indicated contamination with N'-acetimidoglucagon. However, the [des-His']N'-acetimidoglucagon used in the biological assays was purified by cation-exchange chromatography to remove the small amount of deamidated material that may have been generated.

Preparation of N'-tBoc-N'-DNP-L-histidine-ONSu—N'-tBoc-N'-DNP-L-histidine (50 mg; 0.119 mmol) and N-hydroxysuccinimide generated. Procedures were employed to destroy Trp in order to quantitate N”-DNP-His-ONSu in the sample was 53 pmol/ml. TLC analysis of this material, the N”-tBoc-His-ONSu in the sample was 90% formation of active ester as judged by visual inspection of the TLC plate under ultraviolet light, the concentration of N”-tBoc-His-ONSu in the sample was 53 pmol/ml.

The spectra were analyzed by the method of Greenfield and Fasman (1972). The sample was incubated in a nitrogen atmosphere at room temperature in the dark for 30 min, evaporated to the dry state and then lyophilized. The residue was dissolved in 2 ml of dimethylformamide for immediate use in a coupling reaction. Based on 90% formation of active ester as judged by visual inspection of the TLC plate under ultraviolet light, the concentration of N”-tBoc-N'-DNP-L-histidine-ONSu was 50 mg/ml.

Preparation of Semisynthetic N'-Acetimidoglucagon—The coupling of N'-tBoc-N'-DNP-L-histidine-ONSu to [des-His']N'-acetimidoglucagon was carried out under conditions similar to those employed by Saunders and Offord (1977) in coupling various amino acids to insulin. [des-His']N'-acetimidoglucagon (4.6 mg; 1.56 μmol) was dissolved in 1 ml of trifluoroacetic acid in a nitrogen atmosphere. The sample was vortexed immediately and the trifluoroacetic acid was removed rapidly under a stream of nitrogen. To the oily residue was added 840 μl of dimethylformamide containing 10-fold molar excess of 1-hydroxybenzotriazole (2.09 mg; 13.6 μmol) over peptide. Enough N-ethylmorpholine (approximately 10 μl) was added to adjust the apparent pH determined as described by Rees and Offord (1976) to 8-8.5. A 10-fold molar excess of N”-tBoc-N'-DNP-L-histidine-ONSu (7.3 mg; 13.6 μmol) was added to the peptide solution in the appropriate volume (250 μl) of active ester solution prepared as described above. The reaction was stirred in the dark for 12 h while the apparent pH was maintained at 8-8.5 by the addition of 1-2 μl of N-ethylmorpholine.

After the reaction had proceeded for 12 h, the sample was treated at 37°C for 30 min with a 10-fold molar excess of N,N-dicyclohexylcarbodiimide (0.96 ml; 13.6 mmol) over the amount of active ester to remove the DNP group (Shaltiel and Fridkin, 1970; Goren and Fridkin, 1976). These conditions led to essentially complete deprotection of N”-tBoc-N'-DNP-histidine as monitored by the appearance of S-DNP-2-ethylmercaptoethanol, the thiolytic by-product (λ_max = 341 nm; t_{1/2} = 12,500 μm/min) (Shaltiel and Fridkin, 1970). Lower temperatures as also shown. If excess unreacted N,N-dicyclohexylcarbodiimide was added, the reaction was terminated by gel filtration and following lyophilization, the purity of the crude N”-tBoc-semisynthetic derivative was carried out by cation-exchange chromatography on a CM-cellulose column under conditions similar to those used for the purification of native glucagon except that the buffer pH values were 4.5. The major peptide fraction was desalted, the tBoc group was removed and the crude material was purified by cation-exchange chromatography under the conditions given above. An additional cation-exchange purification of [Phel]N'-acetimidoglucagon was required to obtain a homogeneous derivative.

Determination of Extent of Racemization—L-Amino acid oxidase digests were prepared using modifications of procedures of Syrier and Beyerman (1974) and Jones and Ramage (1979). Peptide samples (175 nmol) were hydrolyzed in acid, the sample was evaporated to dryness, the residue was dissolved in water (1 ml) and enough sodium bicarbonate solution was added to adjust the pH to approximately 7 on range 1-11 pH paper. After evaporation, the residue was dissolved in 400 μl of 0.2 M Tris-HCl (pH 7.5). Toluene (5 μl) and 0.4 unit of L-amino acid oxidase in 10 μl of 0.2 M Tris-HCl (pH 7.5) were added to an aliquot which was incubated with stirring at 37°C for 12 h. At this time, a second aliquot of enzyme was added and the incubation continued for another 12 h. Amino acid analyses were performed both before and after oxidation treatment.

Isoelectric Focusing—Isoelectric focusing was carried out on 7.5% polyacrylamide gels (5 x 105 mm) containing 6 M urea and 2% (w/v) pH 3-10 amylolytes as described previously (Enghild et al., 1982). The sensitivity of the Coomassie blue G-250 staining determined by focusing known amounts of native glucagon yielded a detection limit of 1 ng.

Amino Acid Analysis—Rinoxid hydrolysis and enzymatic digests with leucine aminopeptidase were performed and analyzed as described by Flanders et al. (1982).

Circular Dichroism—Circular dichroism measurements were performed as previously described (Rothgeb et al., 1978). The protein concentrations of stock solutions were determined by their absorbance at 278 nm using a molar absorptivity of 3810 M^-1 cm^-1 and diluted with 0.01 N HCl and 2-chloroethanol to 0.3 mg/ml or less. The spectra were analyzed by the method of Greenfield and Fasman (1979) using the reference spectra of Chen et al. (1974) taken at 1-nm intervals over the range 190-250 nm.

Biological Assays—Binding and adenylate cyclase assays were performed as described by England et al. (1982), except that the incubation buffer used in the binding assay was 30 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0.1% bovine serum albumin. Results of the adenylate cyclase assay are expressed as a percentage of activation over basal level which averaged about 0.4 nmol of cAMP formed/mg of membrane protein in 10 min. Maximum activity was 3-4 times the basal level activity. For each set of assays, maximum stimulation of adenylate cyclase by native glucagon was determined for use as a control. In the binding assays, nonspecific binding measured in the presence of 1 μM Trp was 5-10% of specific binding and was subtracted from the total to give specific binding. Results are expressed as the percentage of maximum specific binding which was about 1 x 10^6 cpm/mg of membrane protein. In both assays, the standard deviation for individual points was approximately 5%.
RESULTS

Coupling of N\textsuperscript{\textalpha}-tBoc-N\textsuperscript{\textomega}-L-DNP-L-His-ONSu to [des-His]\textsuperscript{\textalpha} N\textsuperscript{\textalpha}-Acetimidoglucagon—Due to the insolubility of glucagon in organic solvents, initial couplings of histidine were performed in dimethylformamide-H\textsubscript{2}O (3:1). Despite a 24-h coupling and a 50-fold molar excess of active ester at pH 8.8, a disappointing 15% incorporation of histidine occurred even though similar conditions for coupling various amino acids to ferredoxin (Hong and Rabinowitz, 1970; Lode et al., 1976) led to essentially quantitative coupling. In an attempt to increase coupling yields by performing the reactions in a totally organic medium, the [des-His]\textsuperscript{\textalpha} N\textsuperscript{\textalpha}-acetimidoglucagon was solubilized by forming the trifluoroacetate salts of the amines which are liberated by subsequent treatment with N-ethylmorpholine (Bodansky et al., 1967).

1-Hydroxybenzotriazole was included in the coupling reaction (Konig and Geiger, 1972, 1973) to accelerate the rate of active ester coupling of the sterically hindered, doubly protected histidine in dimethylformamide. Even with as much as a 25-fold molar excess of active ester, in the absence of 1-hydroxybenzotriazole the coupling resulted in the incorporation of only 0.77 residue of histidine.

The conditions chosen were optimized to obtain maximum acylation at the NH\textsubscript{\textalpha} terminus with minimum O-acylation which has been reported in some N-hydroxysuccinimide active ester couplings (Slotboom and de Haas, 1975; Bodansky et al., 1977; Dimarchi et al., 1979, 1980). Isoelectric focusing after deprotection showed the major band to focus with N\textsuperscript{\textalpha}-acetimidoglucagon. Minor bands corresponded to [des-His]\textsuperscript{\textalpha} N\textsuperscript{\textalpha}-acetimidoglucagon and the coupling resulted in the incorporation of only 0.77 residue of histidine.

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The alanine and phenylalanine couplings were carried out under conditions essentially identical to those used in the histidine coupling, but were complete with a much shorter reaction time, indicating that steric hindrance was involved in the coupling of the doubly protected histidine.

Purification and Chemical Characterization of Semisynthetic N\textsuperscript{\textalpha}-Acetimidoglucagon—As shown in Fig. 1, a major peak (IV) corresponding to N\textsuperscript{\textalpha}-acetimidoglucagon by amino acid analysis (Table I) could be separated from other peaks (I and II) which result from the use of the anisole and dithiothreitol in the deprotection by trifluoroacetic acid and from the unreacted [des-His]\textsuperscript{\textalpha} N\textsuperscript{\textalpha}-acetimidoglucagon (peak III). The treatment of native glucagon with trifluoroacetic acid under these conditions did not affect its elution position from the cation-exchange column.

Isoelectric focusing of the once purified semisynthetic material showed that a major product focused with N\textsuperscript{\textalpha}-acetimidoglucagon prepared from native glucagon (pH 6.5). Two minor bands (~5% of total material) were seen; one at the position of [des-His]\textsuperscript{\textalpha} N\textsuperscript{\textalpha}-acetimidoglucagon and the other at a position more positive than native glucagon such as would be expected with O-acylation. A second identical cation-exchange purification removed these contaminants. The overall yield of semisynthetic hormone after two cation-exchange purifications starting from purified native glucagon is 5–10%.

The results of amino acid analysis of material from peak IV following acid hydrolysis are shown in Table I. The expected number of residues of Thr, Ser, and Tyr were recovered upon leucine aminopeptidase digestion of the derivatives with leucine aminopeptidase.

| Amino acid | Authentic N\textsuperscript{\textalpha}-acetimidoglucagon | Semisynthetic N\textsuperscript{\textalpha}-acetimidoglucagon | [Ala']N\textsuperscript{\textalpha}-acetimidoglucagon | [Phe']N\textsuperscript{\textalpha}-acetimidoglucagon |
|------------|---------------------------------|-----------------|-----------------|-----------------|
| Asx (4)    | 3.84a                          | 3.59            | 3.93            | 3.93            |
| Thr (3)    | 2.93a                          | 2.90b           | 2.92b           | 2.91b           |
| Ser (4)    | 3.87a                          | 4.10b           | 4.07b           | 4.09b           |
| Gly (4)    | 3.08                           | 3.10            | 3.14            | 2.92            |
| Ala (1)    | 1.07                           | 1.19            | 1.96            | 1.10            |
| Val (1)    | 0.94                           | 1.03            | 0.95            | 0.98            |
| Met (1)    | 1.00                           | 1.00            | 0.93            | 1.08            |
| Leu (2)    | 1.90                           | 1.97            | 1.92            | 1.95            |
| Tyr (1)    | 2.80                           | 1.97            | 1.97            | 1.97            |
| Phe (1)    | 2.05                           | 1.92            | 2.00            | 2.97            |
| Lys (0)    | <0.02a                         | <0.01'          | 0.01'           | 0.01'           |
| His (1)    | 0.92a                          | 0.98'           | 0'              | 0'              |
| Arg (2)    | 2.07a                          | 2.04'           | 2.09'           | 2.08'           |
| Trp (1)    | 1.11a                          | 0.98'           | 0.91'           | 0.92'           |

* Expected values in parentheses are based on the glucagon sequence.

The values were obtained from analysis on the lithium citrate column following digestion of the derivatives with leucine aminopeptidase.

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Semisynthetic [Ala']- and [Phe']N'-Acetimidoglucagon

Fig. 2. Purifications by CM-cellulose cation-exchange chromatography. Top, elution profile for the purification of crude [tBoc-Ala']N'-acetimidoglucagon. Bottom, elution profile for the purification of crude [Ala']N'-acetimidoglucagon. Details are given in the text. —, absorbance, 278 nm; ——, conductivity in millimhos. Similar elution profiles were obtained for the purification of [tBoc-Phe']- and [Phe']N'-acetimidoglucagon using identical conditions.

A third identical cation-exchange purification yielded a single component on isoelectric focusing of 100 μg of sample.

Determination of Extent of Racemization—In control studies, l-amino acid oxidase treatment destroyed 99.8% of L-His and 1.87% D-Phe/Phe residue. The semisynthetic N'-acetimidoglucagon sample contained 2.1% D-His while [Phe']N'-acetimidoglucagon showed 1.70% D-Phe/Phe residue. These results indicate that essentially no racemization of the coupled amino acids had occurred during the extensive procedures necessary for its preparation. No racemization would be expected with [Ala'] N'-acetimidoglucagon as it was prepared identically to the [Phe'] derivative and alanine shows no enhanced propensity to racemize.

Circular Dichroism Analysis of Derivatives—The CD spectra of dilute solutions of the modified derivatives obtained in 0.01 N HCl and various concentrations of the helix-forming solvent, 2-chloroethanol, are shown in Fig. 3. As expected, all derivatives demonstrated an increase in α-helicity in this solvent (Gratzer and Beaven, 1969; Srere and Brooks, 1969; Epand, 1972a). In 0.01 N HCl in the absence of 2-chloroethanol, the percentage of β-sheet content was approximately equivalent to that of α-helical content for all peptides while in 2-chloroethanol no β-structure was found and the secondary structure was composed entirely of α-helix and random coil. Also in the absence of 2-chloroethanol in 0.01 N HCl native glucagon, N'-acetimidoglucagon and [Phe']N'-acetimidoglucagon exhibited 13~15% α-helix, while surprisingly [Ala']N'-acetimidoglucagon and [des-His']N'-acetimidoglucagon showed almost 30% helicity. The concentrations used (approximately 0.3 mg/ml) were more dilute than that at which an increase in helicity is observed due to the aggregation of native glucagon into trimers which stabilize the α-helical configuration (Epand, 1972b; Panijpan and Gratzer, 1974). The concentration of [Phe']N'-acetimidoglucagon studied after the L-amino acid oxidase treatment showed the native glucagon sample to contain 2.0% D-His and 1.87% D-Phe/Phe residue. The semisynthetic N'-acetimidoglucagon sample contained 2.1% D-His while [Phe']N'-acetimidoglucagon showed 1.70% D-Phe/Phe residue. These results indicate that essentially no racemization of the coupled amino acids had occurred during the extensive procedures necessary for its preparation. No racemization would be expected with [Ala'] N'-acetimidoglucagon as it was prepared identically to the [Phe'] derivative and alanine shows no enhanced propensity to racemize.

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was only 0.18 mg/ml due to its limited solubility even with warming.

To determine if the higher helical content of [Ala']N'-acetimidoglucagon, as compared with native glucagon, was due to aggregation to trimers, the CD spectra of diluted samples of native glucagon, [Phe']N'-acetimidoglucagon and [Ala']N'-acetimidoglucagon in 0.01 N HCl were obtained. The α-helical content of the [Ala'] derivative was found to decrease with dilution while the native glucagon and [Phe'] analogue did not display this phenomenon. This suggests that the higher helical content of [Ala']N'-acetimidoglucagon at 0.3 mg/ml may be due to the self-association of the derivative into trimers with a concomitant increase in helicity.

**Biological Characterization of Glucagon Derivatives**—Both N'-acetimidoglucagon prepared from native hormone and twice-purified semisynthetic N'-acetimidoglucagon were essentially identical with native glucagon in their abilities to activate adenylate cyclase and to bind to rat liver plasma membranes as shown in Fig. 4, A and B, respectively. The peptide concentration giving half-maximal activation was 2.1 \( \times 10^{-9} \) M for native glucagon, 1.9 \( \times 10^{-9} \) M for N'-acetimidoglucagon, and 2.4 \( \times 10^{-9} \) M for semisynthetic N'-acetimidoglucagon, while the concentration displacing 50% of mono\([^{125}\text{I}]\)iodoglucagon from membranes was 1.4 \( \times 10^{-9} \) M for native glucagon, 1.4 \( \times 10^{-9} \) M for N'-acetimidoglucagon, and 2.3 \( \times 10^{-9} \) M for semisynthetic N'-acetimidoglucagon. All values have an error of \( \pm 1 \times 10^{-9} \) M. The half-maximal binding and activation values previously reported from this laboratory for N'-acetimidoglucagon were approximately 50% of those for native glucagon (Flanders et al., 1982). The closeness of the values reported here probably result from the cation-exchange purification added in the preparation of N'-acetimidoglucagon which removes an impurity that may have been present in the first preparation.

Fig. 4, A and B, also shows the activation and binding exhibited by [des-His']N'-acetimidoglucagon. The concentration of derivative displacing 50% of mono\([^{125}\text{I}]\)iodoglucagon from membranes was 3.5 \( \times 10^{-8} \) M, giving a relative binding affinity of 4.0% while the concentration required for half-maximal activation was 3.3 \( \times 10^{-8} \) M, giving a relative biological potency of 5.7% when compared with N'-acetimidoglucagon. As can be seen in Fig. 4A, this derivative is a partial agonist, attaining only 35% of the maximum activation elicited by native glucagon and the other derivatives. This is in accord with other reports (Lin et al., 1975; Bregman and Hruby, 1979) in which preparations of [des-His']glucagon were found to be partial agonists, but the degree of maximum activation is more than in our more highly purified preparation, perhaps due to slight contamination with fully active native glucagon.

A separate series of adenylate cyclase activation and receptor-binding assays were performed to characterize [Ala']- and [Phe']N'-acetimidoglucagon and the results are shown in Fig. 5, A and B, respectively. Table II reports the derivative concentrations required for half-maximal activation and displacement of mono\([^{125}\text{I}]\)iodoglucagon bound to membranes along with those values for N'-acetimidoglucagon and native glucagon assayed for control. Since the derivatives were partial agonists, Table II also gives the per cent maximal activation as compared to N'-acetimidoglucagon. All derivatives exhibited high dose inhibition (Englund et al., 1983), ensuring that maximal stimulation was attained. These points were not included in computer fits of the data which were used to calculate the half-maximal activation concentration and per cent maximal activity.

**DISCUSSION**

The regeneration of fully active N'-acetimidoglucagon and the preparation of [Ala']- and [Phe']N'-acetimidoglucagon have been accomplished. The coupling of histidine posed special problems due to the limited solubility of glucagon, the slow rate of coupling of the sterically hindered histidine active ester, and the susceptibility of histidine to racemization which necessitates the use of both N° and N" protection. The solubilization of [des-His']N'-acetimidoglucagon in dimethylformamide and the use of 1-hydroxybenzotriazole as a catalyst (Konig and Geiger, 1972, 1973; Saunders and Offord, 1977; Geiger et al., 1978; Kisfaludy, 1979) are required for the quantitative coupling of the sterically hindered histidine in a reasonable time.

Protection of the imidazole nitrogen of histidine with DNP (Henkart, 1971) virtually eliminates its racemization (Erickson and Merrifield, 1976; Bodansky et al., 1976). Furthermore, the thiolytic protection conditions are sufficiently mild to avoid additional damage to the molecule (Salaitel and Fridkin, 1970). The use of the tBoc group prevents undesired racemization, presumably by eliminating oxazolinone formation (Erickson and Merrifield, 1976; Bodansky et al., 1976; Barany and Merrifield, 1979) as well as acylation. These protecting groups proved effective as only 0.1% D-histidine was detected in the semisynthetic peptide.

**Structural Studies**—Although the substitutions at the amino-terminal residue would not, a priori, be expected to alter the secondary structure of the peptide (Chou and Fas-
propensity to self-associate. A ring system at the side chain of position 1 may provide enough steric hindrance to make β-turn formation difficult, thereby prohibiting the formation of a greater degree of secondary structure.

In the presence of 2-chloroethanol, all of the peptides increased in α-helix content (Beaven et al., 1969) with a concomitant decrease in β-sheet content, indicating that the structure at the amino terminus does not affect this phenomenon. Thus, the increase in helicity in organic solvents which is not associated with self-association differs markedly from the increased extent of helicity of the [Ala'] derivative which appears to be due to trimerization.

Functional Studies—Biological characterization of the [des-His'], [Ala'] and [Phe'] derivatives showed them to be partial agonists with each analogue exhibiting comparable decreases in binding and activation potencies. [des-His'] N'-acetimidogluca
gon transduces poorly either because of the loss of the side chain or of the displacement of the α-amino group as a result of shortening the peptide. However, the [Ala'] derivative with an α-amino group in the original location has greater transducing ability than the [des-His'] analogue, suggesting that the position of the α-amino group is important in transduction. Moreover, the side chain of the residue at position 1 must also be involved in transduction since, as the side chain is replaced by the methyl group of Ala', the nonhydrogen-bonding, planar, aromatic ring system of Phe' and ultimately, the aromatic, hydrogen-bonding imidazole ring, the maximum activation of adenylate cyclase increases.

A large aromatic ring system at the side chain of the amino-terminal residue also seems to be very important for proper receptor binding, as much as the [Phe'] derivative shows only a slight reduction in binding ability when compared to N'-acetimidogluca
gon and native glucagon. Paradoxically, [Ala'] N'-acetimidogluca
gon shows less potency than the [des-His'] analogue. Perhaps, the hydrogen-binding capability of the side chain of either His-1 or Ser-2 also contributes to the binding potential. Clearly, some compensatory mechanism of receptor interaction must be present in the [des-His'] derivative which, even in the absence of the first residue, allows it to bind with a higher affinity than the [Ala'] derivative.

Although formal analyses were not attempted, the slopes of the binding curves of the derivatives appear to be similar to those of native glucagon and other derivatives modified throughout the molecule (England et al., 1983), suggesting that these derivatives, like those studied previously, interact with the receptor in a noncooperative manner. Furthermore, at high concentrations, inhibition of adenylate cyclase activity is characteristic of these derivatives. As pointed out previously

| Peptide                  | Half-maximal activation concentration* | Relative maximal displacement concentration | Relative binding affinity* | Maximal activity* |
|-------------------------|----------------------------------------|--------------------------------------------|---------------------------|------------------|
| Native glucagon         | 1.01 nM                                 | 1.45                                       | 100%                      | 100%             |
| N'-Acetimidogluca
gon | 1.38 nM                                 | 100%                                       | 100%                      | 100%             |
| [Phe']N'-acetimidogluca
gon | 5.07 27.2                               | 7.71                                       | 22.4%                     | 86%              |
| [Ala']N'-acetimidogluca
gon | 67.8 2.0                                 | 108%                                      | 1.6%                      | 55%              |

*Average of two or three separate assays.
*Relative biological potency equals (N'-acetimidogluca
gon concentration required for half-maximal activation)/(derivative concentration required for half-maximal activation) x 100.
*Relative binding affinity equals (N'-acetimidogluca
gon concentration required for half-maximal displacement of the 125I-glucagon)/(derivative concentration required for half-maximal displacement of the 125I-glucagon) x 100.

Fig. 5. Biological characterization of derivatives with alternative residues at position 1. A, dose-response curves for the activation of rat liver adenylate cyclase. B, displacement of monol[125I]iodoglucagon bound to rat liver plasma membranes. Points are the mean of duplicate determinations. O, N'-acetimidogluca
gon; △, [Ala']N'-acetimidogluca
gon; □, [des-His']N'-acetimidogluca
gon.
(England et al., 1983), these features differentiate glucagon-receptor interactions from those of the adrenergic system and must be taken into account in the development of models of the glucagon-receptor system.

The results presented here indicate that the α-amino group of glucagon is involved to a greater extent in the transduction of adenylate cyclase than in the binding process. The imidazole moiety of histidine-1 participates directly with the results of the biological assays, they do indicate that the amino-terminal substitutions may cause unexpected structure changes. Clearly, the semisynthetic peptides and proteins (Offord, R. E., and DiBello, C., eds) pp. 141-159, Academic Press, New York

Epond, R. M. (1972a) J. Biol. Chem. 247, 2132-2138
Epond, R. M. (1972b) Arch. Biochem. Biophys. 148, 325-326
Epond, R. M., and Wheeler, G. E. (1975) Biochim. Biophys. Acta 393, 236-246
Epond, R. M., Rosselin, G., Hoa, D. H. B., Cote, T. E., and Laburthe, M. (1981) J. Biol. Chem. 256, 1128-1132
Erickson, B. W., and Merrifield, R. B. (1976) in The Proteins (Neurath, H., and Hill, R. L., eds) pp. 255-257, Academic Press, New York

Flanders, K. C., Mar, D. H., Fozz, R. J., England, R. D., Coolican, S. A., Harris, D. E., Floyd, A. D., and Gurs, R. S. (1982) Biochemistry 21, 4244-4251
Garner, W. H., and Gurd, F. R. N. (1975) Biochem. Biophys. Res. Commun. 63, 262-268
Geiger, R., Teetz, V., König, W., and Obermeier, R. (1978) in Semisynthetic Peptides and Proteins (Offord, R. E., and DiBello, C., eds) pp. 141-159, Academic Press, New York

Goren, H. J., and Fridkin, M. (1978) Int. J. Peptide Protein Res. 11, 1-8
Gratzner, W. B., and Beaven, G. H. (1969) J. Biol. Chem. 244, 6675-6679
Greenfield, N., and Fasmou, G. D. (1969) Biochemistry 8, 4108-4116
Greenstein, J. P., Birnbaum, S. M., and Oste, M. C. (1963) J. Biol. Chem. 204, 307-321
Henkart, P. F. (1971) J. Biol. Chem. 246, 2711-2713
Hing, J.-S., and Rabinowitz, J. C. (1979) J. Biol. Chem. 245, 4988-4994
Jones, J. H., and Ramage, W. I. (1979) Int. J. Peptide Protein Res. 14, 65-67
Kisfaludy, L. (1979) in The Peptides: Analysis, Synthesis, Biology (Gross, E., and Meienhofer, J., eds) Vol. 2, pp. 417-440, Academic Press, New York
König, W., and Geiger, R. (1972) in Chemistry and Biology of Peptides (Meienhofer, J., ed) pp. 343-350, Ann Arbor Science Publishers, Ann Arbor, MI
König, W., and Geiger, R. (1973) Chem. Ber. 106, 3626-3635
Lewis, P. N., Mermay, F. A., and Scheraga, H. A. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 2293-2297
Liepinies, J. J., and Epond, R. M. (1982) Biochim. Biophys. Acta 707, 171-177
Lin, M. C., Wright, D. E., Hruby, V. J., and Rodbell, M. (1975) Biochemistry 14, 1559-1563
Lode, E. T., Murray, C. L., Sweeney, W. V., and Rabinowitz, J. C. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1361-1365
Lode, E. T., Murray, C. L., and Rabinowitz, J. C. (1976) J. Biol. Chem. 251, 1675-1682
Lundt, B. F., Johansen, N. L., Volund, A., and Markussen, J. (1978) Peptide Protein Res. 14, 255-263
Nakajima, K., and Okawa, K. (1973) Bull. Chem. Soc. Jpn. 46, 8411-8416
Neuberger, A. (1948) Adv. Protein Chem. 4, 297-382
Pantipiti, B., and Gratzner, W. B. (1974) Eur. J. Biochem. 45, 547-552
Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1969) Science (Wash. D. C.) 164, 566-569
Rees, A. R., and Offord R. E. (1976) Biochem. J. 159, 467-486
Rodbell, M., Birnbaumer, L., Pohl, S. L., and Sandby, F. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 909-913
Rothgeb, T. M., England, R. D., Jones, B. N., and Gurd, R. S. (1978) Biochemistry 17, 4564-4571
Saunders, D. J., and Offord, R. (1977) Biochem. J. 165, 479-486
Shaltiel, S., and Fridkin, M. (1970) Biochemistry 9, 5122-5127
Slotboom, A. J., and de Haas, G. H. (1975) Biochemistry 14, 5394-5399
Slotboom, A. J., van Dam-Mieras, M. C. E., and de Haas, G. H. (1977) J. Biol. Chem. 252, 2946-2951
Slotboom, A. J., Jansen, E. H. J. M., Pattus, F., and de Haas, G. H. (1975) in Semisynthetic Peptides and Proteins (Offord, R. E., and DiBello, C., eds) pp. 315-348, Academic Press, New York
Sreer, P. A., and Brooks, G. C. (1969) Arch. Biochem. Biophys. 129, 708-710
Syrjä, J. L. M., and Beyerman, H. C. (1974) Recueil. J. R. Neth. Chem. Soc. 93, 117-120
Weinre, V. M., Brandenburg, D., and Zahn, H. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1556-1562
Yeung, C. W. T., Moule, M. L., and Yip, C. C. (1979) J. Biol. Chem. 254, 9439-9457