The Relation of Predicted Structure to Observed Conformation and Activity of Glucagon Analogs Containing Replacements at Positions 19, 22, and 23*

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Joseph Murphy, Wei-Jun Zhang, William Macaulay, Gerald Fauman†, and R. B. Merrifield§

From The Rockefeller University, New York, New York 10021 and Brandeis University, Waltham, Massachusetts 02254

Six new analogs of glucagon have been synthesized containing replacements at positions 19, 22, and 23. They were designed to study the correlation between predicted conformation in the 19–27 segment of the hormone and the conformation calculated from circular dichroism measurements and the observed activation of adenylate cyclase in the liver membrane. The analogs were [Val19]glucagon, [Val22]glucagon, [Glu23]glucagon, [Val19,Val22]glucagon, [Glu22,Glu23]glucagon, and [Ala22,Ala23]glucagon. The structures predicted for the 19–27 segment ranged from strongly α helical to weakly β sheet. The observed conformations varied as functions of amino acid composition, solvent, concentration, pH, and temperature but did not correlate well with prediction. There was, however, a correlation between predicted structure and activation of adenylate cyclase in rat liver membranes.

Glucagon is a 29-residue peptide (1) formed in the A cells of pancreatic islets by posttranslational processing of a large precursor protein (2, 3). It was originally recognized as a hyperglycemic factor (4) and later shown to function by binding to specific liver plasma membrane receptors that are coupled via GTP-dependent regulatory proteins to the activation of membrane-bound adenylate cyclase (5). This enzyme in turn catalyzes conversion of adenosine triphosphate (ATP) to cyclic 3',5'-adenosine monophosphate (cAMP), which affects key enzymes in the glycogenolysis and gluconeogenesis pathways leading to elevated blood glucose (6).

Our objective has been to synthesize analogs of glucagon having predictable changes in conformation that can be correlated with strength of binding of the hormone to hepatocyte receptors and with the transduction of the signal leading to production of the cAMP second messenger.

The x-ray crystal structure of glucagon is known (7), but the conformation of the molecule in dilute solution in the circulation or after binding to its receptor is not known. Some indications of structure have been obtained by circular dichroism measurements (8–11) and by NMR studies (12), and Chou and Fauman (13) have predicted the probable shape of the hormone in solution based on their scale of conformational potentials of amino acid residues to form an α helix, β sheet, or γ turn (14). The latter data were derived from proteins of known crystal structure, and the assumption was made that amino acid residues in a small peptide like glucagon might behave in the same way. The analysis showed that glucagon should have a β turn at residues 2–5, a β sheet from 5–10, turns at 10–13 and 15–18, and a region from 19 to 27 that is balanced closely between a sheet and a helix (13). It was predicted that substitution of 1 or 2 residues in this region by residues strongly favoring one of these conformations would be enough to change the entire 19–27 segment into a single conformation and that circular dichroism measurements would be sensitive enough to detect such a large change. Thus, they predicted that [Glu22]glucagon would favor the helical structure and that a double substitution of the normal Phe22, Val23 sequence by Glu22–Glu23 would lock the 19–27 region into an α helix while the β sheet structure would be very unlikely statistically. They also proposed that the β conformer would probably be required for receptor binding and that the Glu22–Glu23 analog would bind poorly and be inactive. In an effort to test this hypothesis and to understand the role of conformation in the binding of glucagon to its hepatocyte receptors and in the transduction of the hormonal signal, we have synthesized a series of analogs containing amino acid replacements at positions 19, 22, and 23. These were chosen to favor either α helices or β sheets in order to be able to relate predicted conformation with structure calculated from circular dichroism measurements. If the applicability of the Chou-Fauman predictive rules to glucagon could be established, we should be able to construct analogs which bind tightly to the hepatocyte receptor and function either as superagonists or antagonists of glucagon action in the liver cell. The latter should reduce the hyperglycemia of diabetes. This kind of inhibitor would differ from an inhibitor of the release of glucagon from the A cell, such as somatostatin, and should be of considerable value in unraveling the details of the mode of action of glucagon.

EXPERIMENTAL PROCEDURES

Materials—Copoly(styrene-1%-divinylbenzene) resin, 200–400 mesh, (Bio-Rad) was washed, sized, and converted to aminomethyl mesh, (Bio-Rad) was washed, sized, and converted to aminomethyl

1. The abbreviations used are: Boc-[³H]Leu, t-butyloxycarbonyl[³H] leucine; TFE, trifluoroethanol; Bzl, benzyl; Tos, tosyl; DMF, N,N-dimethylformamide; HPLC, high performance liquid chromatography; For, formic; HOBr, N'-hydroxybenzotriazole; Ace, tetramethylxycarbonyl; CI, 2-chlorobenzyloxycarbonyl; BrZ, 2-bromobenzoxycarbonyl; Dnp, 2,4-dinitrophenyl; cHx, cyclohexyl; OMPA, oxymethylxycarbonyl acid.

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† To whom correspondence should be addressed: The Rockefeller University, 1230 York Avenue, New York, NY 10021.
mCi/mmol, were prepared from the radiolabeled amino acids (Du Pont, New Haven, Conn.) by acylation with di-tert-butyl pyrocatecholate (17). Boc-amino acids were from Peninsula Laboratories, Belmont, CA. The sources and purification of all other reagents for peptide synthesis were the same as described previously (18).

All components for the adenylyl cyclase assay were obtained from Sigma. The ATP was prepared synthetically by phosphorylation of adenine nucleotide prior to minimize contamination by unidentified levels of GTP present in isolated ATP (19). Commercial glucagon (Sigma) was purified on a Ca reverse-phase column before use. Cyclic AMP binding protein assay kits were from Amersham Corp.

Binding of radiolabeled glucagon to purified plasma membranes were isolated by the method of Neville through the extinction coefficients, confirmed by the previously determined specific activity of 3H and 14C in the peptides.

Cyclic AMP was hydrolyzed in 12 N HCl/phenol/HOAc (2:1:1), 110 °C, 24 h, in evacuated tubes. Free peptides were hydrolyzed in 6 N HCl, or in 4 N methanolic sulfuric acid containing 0.2% of 3(2-aminophenyl)indole (Pierce).

Isolation of Liver Plasma Membranes—Partially purified rat liver plasma membranes were isolated by a method of Neville through step 1 (20). After resuspension in an equal volume of ice-cold 1 mM NaHCO₃, membranes were divided into 100-µl aliquots, which were frozen rapidly and stored in liquid nitrogen. Aliquots were thawed immediately before use and never refrozen. For the adenylyl cyclase assay, the protein concentration was adjusted to 0.7 mg/ml as determined by a modified Lowry procedure (21).

Adenylyl Cyclase Assay—Stock solutions of purified natural glucagon and the synthetic peptides were prepared fresh by stirring 0.2-0.5 mg of peptide with 2 ml of 25 mM Tris-HCl, 1 mg/ml bovine serum albumin buffer, pH 7.75, at 24 °C for 1 h. After Millipore filtration, the peptide concentration was determined from absorbance at 276 nm and specific radioactivity. Less concentrated solutions were obtained by serial dilution in buffer. The assay solution was prepared according to the general procedure of Salomon et al. (19). Incubation media were prepared by mixing 40 µl of assay solution, 40 µl of peptide solution, and 20 µl of membrane suspension. After 5 min at 30 °C, the reaction was quenched in a boiling water bath, rapidly cooled in ice, and centrifuged at 12,000 rpm.

The cyclic AMP in the supernatant was quantitated using a commercial assay kit from Amersham Corp., which is based on the conversion of labeled cyclic AMP and a fixed quantity of added cyclic [8-3H]AMP for a specific high affinity cyclic AMP-binding protein, with removal of unbound nucleotides by adsorption on charcoal (22). The cAMP levels to be measured were adjusted to fall in the 1-5-pmol range. The basal activity of different membrane preparations was in the range of 35–45 pmol/mg of protein/min and the response to excess glucagon gave increases in cAMP of 3- to 4-fold. To compensate for these variations, the responses were converted to percent of maximal activation above the basal level (23).

Receptor Binding Assay—The binding of analogs to receptors of the rat liver membrane was measured by displacement of [125I]glucagon according to Wright and Rodbell (24).

RESULTS

The Choice of Analogs—To test the hypothesis that the statistical predictive rules of conformational preference of amino acids derived from crystal structures of proteins would be applicable to a small flexible peptide like glucagon, we followed the suggestion of Chou and Fasman (15). The idea was to make sequence replacements in the 19-27 region of glucagon that would be expected to make significant changes in the conformation of that peptide segment as measured by circular dichroism. Table I shows the quantitative and qualitative α helical and β sheet potentials of residues 19-27 of glucagon and of six selected replacement analogs, and the prediction of the overall conformation of this region. These analogs range from examples strongly predicted to be helical to those predicted to favor the β sheet structure in the 19-27 region.

Synthesis of the Analogs—Three of the analogs, [Glu², Gln²], [Ala², Ala³], and [Val¹]glucagon, were prepared by anchoring COOH-terminal Boc-Thr(Bzl) to chloromethyl copoly(styrene-1%-divinylbenzene) resin support (25) and the other three analogs, [Val¹], [Glu²], and [Val¹,Glu²]glucagon, were prepared on a resin containing an internal reference amino acid (26). Thus, the 1st residue was attached to oxymethylphenacylaminocarboxylic acid (OCH₃-Pan-am resin) (Fig. 1). The attachment of COOH-terminal Boc-Thr(Bzl) to the latter support was carried out as follows (with quantitative ninhydrin values (27) given in parentheses): (a) 6.15 g of aminoethyl-resin, 0.25 mmol/g, swollen in 100 ml of CH₃Cl₂ and coupled for 16 h with 2.4 eq each of Boc-norleucine and dicyclohexylcarbodiimide in CH₃Cl₂ (0.0037 mmol/g of free NH₂ groups unreacted); (b) acetylation of residual amine with Ac₂O/pyridine, 1:1 (0.0001 mmol of NH₂/g); (b) Boc deprotection with CF₃COOH/CH₃Cl₂, 1:1 (ninhydrin, 0.25 mmol/g); (d) 3-h coupling with 1.5 eq each of Boc-Thr(Bzl)-oxymethylphenylacetic acid and dicyclohexylcarbodiimide in CH₃Cl₂ (0.0023 mmol of NH₂/g); (e) 3-h second coupling with 0.3 eq of preformed symmetric anhydride of Boc-Thr(Bzl)-oxymethylphenylacetic acid in CH₃Cl₂ (0.0007 mmol of NH₂/g); (f) acetylation (0.0001 mmol of NH₂/g). The loading was 0.22 mmol/g by picrate titration (28) of a deprotected sample.

The side chain protecting groups were Ser(Bzl), Thr(Bzl), Glu(OBzl), Asp(OBzl) or Asp(OeOH), Lys(2CIZ), Tyr(2BrZ), Trp(For), Arg(Tos), His(Tos) for all analogs except for a second synthesis of [Glu², Gln²]glucagon in which Nε-aminotriphenylsulphonium chloride was used. The Nε-protecting group was t-butylxycarbonyl (Boc) which was removed at each step by 50% trifluoroacetic acid in CH₂Cl₂. The free α-NH₂ was liberated with 5% disopropylethylamine in CH₂Cl₂. The synthetic protocols were essentially as described previously (29). Arginine was coupled with dicyclohexylcarbodiimide in CH₂Cl₂, asparagine and glutamine were coupled as N'-hydroxybenzotriazolone esters made in situ with dicyclohexylcarbodiimide in DMF/CH₂Cl₂ (2:1), and all other residues were coupled as preformed symmetric anhydrides in CH₂Cl₂ except when they were added to glutamine, in which case the solvent was DMF. Three eq of activated derivative were used for each coupling and, after a wash with tertiary amine, a second coupling was routinely carried out. For the anhydride reactions, the second coupling was in DMF in all cases except as noted. For a few residues, a third coupling was needed.

Radiolabeled amino acids were incorporated into all of the peptides to aid in purification. For that purpose [1H]Leu26 and [14C]Gly4 were used.

Monitoring the Syntheses—All syntheses were monitored for completion of reaction by the quantitative ninhydrin reaction (27). Table II shows data for one of the analogs, [Val¹]glucagon, and the results are typical of all the syntheses. In most instances, no difficulty was encountered in reducing residual amino groups below 1 µmol/g of peptide resin. However, to achieve this level for Aoc-Arg(Tos), the coupling time was extended from 2 to 5 hours. Special difficulty was observed in coupling Boc-glutamine at positions 24, 20, and 3. For the first two, a third coupling in N-methylpyrrolidinone solved the problem. In the early stages of this synthesis, the swelling of the resin was 30% greater in N-methylpyrrolidinone than in DMF or CH₂Cl₂ and was probably responsible for the improved coupling. Beyond the undecapeptide stage, swelling in DMF and CH₂Cl₂ began to increase significantly.

The deprotected resin was also monitored by the ninhydrin
Conformation and Activity of Synthetic Glucagon Analogs

TABLE I
Predicted conformations of glucagon analogs

| Peptide | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | Average potential | Predicted conformation |
|---------|----|----|----|----|----|----|----|----|------------------|-----------------------|
| I Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.18 |
| II [Val^{22},Val^{23}]Glucagon | Val | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.14 |
| III [Val^{22},Val^{23}]Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.17 |
| IV [Tyr^{22}]Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.19 |
| V [Val^{22},Val^{23}]Glucagon | Val | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.10 |
| VI [Glu^{22}]Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.23 |
| VII [Ala^{22},Ala^{23}]Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 0.95 |
| VIII [Glu^{22},Glu^{23}]Glucagon | Ala | Gln | Asp | Phe | Val | Gln | Trp | Leu | Met | P_α | 1.27 |

Fig. 1. The sequence of protected [Glu^{23}]glucagon-OCH_{2}-Pan-am resin. The other analogs had replacements at positions 19, 22, or 23 but contained the same protecting groups and radiolabels. The resin support for [Val^{22}] and [Val^{23},Glu^{23}]glucagon was the same, but for [Glu^{23},Glu^{22}]-, [Ala^{23},Ala^{23}]-, and [Val^{22}]glucagon the phenylacetylenorleucylaminomethyl linker was omitted. @ represents the copoly(styrene-1%-divinylbenzene) resin bead.

reaction at each cycle of the synthesis (Fig. 2). The data were corrected for the gain in weight due to the growing peptide chain and are reported as millimoles of NH_{2}/g of polystyrene. Significant drops in amine occurred following incorporation of Boc-glutamine at residues 24 and 20, indicating about 20% loss of growing chains. Since radiolabel data showed clearly that there was no loss of peptide chains from the resin, the decrease in growing chains is attributed to chain termination. This was correlated with a 34% decreased incorporation of [^{14}C]glycine at position 4 near the end of the synthesis relative to [^{3}H]leucine at residue 26 at the beginning of the synthesis. When the ninhydrin-monitoring data were corrected for such chain termination (Fig. 2), the calculated curve and observed data points agree reasonably well. Such chain termination at glutamine was not observed in previous glucagon syntheses, and we have no good explanation for the effect, although an ultraviolet absorbing contaminant in our commercial Boc-glutamine has been found and may be responsible for the problem.

Cleavage and Purification of the Analogs—The two-step "low/high HF" procedure (30) was used for cleavage of all peptides. This process simultaneously removed all protecting groups, including the formyl on tryptophan, and reduced any methionine sulfoxide produced during the synthesis back to methionine. After evaporation of HF and dimethyl sulfide, the residual scavengers and by-products were extracted with ether and then the crude peptides were extracted into 10% aqueous acetic acid. The solution was dialyzed against several changes of 10% acetic acid and lyophilized.

Analytical HPLC of the crude [Val^{23}]glucagon revealed the peptide peaks shown in Fig. 3. Repetition of the HF cleavage
Ninhydrin monitoring of [Val$^{33}$]glucagon synthesis

| Residue coupled | Amino group remaining | Coupling (corrected)* |
|-----------------|-----------------------|-----------------------|
|                 | 1st | 2nd | Last | µmol/g | %     |
| 29 Thr(Bz)OMPA  | 1.63 | 0.65 | 99.9 |
| 28 Aan          | 0.63 | 0.34 | 100  |
| 27 Met          | 1.71 | 0.18 | 100  |
| 26 [H]Leu       | 0.77 | 99.9 |
| 25 Trp(Fos)     | 0.78 | 99.9 |
| 24 Gln          | 0.62 | 99.9 |
| 23 Val          | 0.71 | 99.9 |
| 22 Phe          | 0.82 | 99.9 |
| 21 Asp(OBz)     | 0.61 | 99.9 |
| 20 Gln          | 3.00 | 0.45 | 100  |
| 19 Val          | 1.17 | 0.90 | 99.6 |
| 18 Arg(Tos)     | 0.59 | 99.2 |
| 17 Arg(Tos)     | 1.17 | 0.90 | 99.6 |
| 16 Ser(Bz)      | 0.44 | 100  |
| 15 Asp(Ochx)    | 1.00 | 99.5 |
| 14 Leu          | 1.06 | 0.53 | 100  |
| 13 Tyr(BrZ)     | 1.20 | 0.80 | 99.7 |
| 12 Lys(Ciz)     | 0.85 | 99.6 |
| 11 Ser(Bz)      | 0.65 | 99.8 |
| 10 Tyr(BrZ)     | 0.73 | 99.7 |
| 9 Asp(OBz)      | 0.10 | 100  |
| 8 Ser(Bz)       | 0.53 | 100  |
| 7 Thr(Bz)       | 1.03 | 0.84 | 99.6 |
| 6 Phe           | 0.60 | 99.9 |
| 5 Thr(Bz)       | 1.11 | 0.46 | 100  |
| 4 [C]Gly        | 0.51 | 100  |
| 3 Gln           | 1.74 | 98.2 |
| 2 Ser(Bz)       | 1.90 | 1.26 | 98.7 |
| 1 His(Tos)      | 2.59 | 1.37 | 98.0 |

*The final ninhydrin value is divided by the amount of growing chain remaining at each cycle of the synthesis, which is determined by ninhydrin analysis of the unprotected peptide-resin. These values have been corrected for a background of 0.50 µmol/g, which is always seen in these assays. Its origin is uncertain, but it is presumed to be due to ninhydrin-positive impurities in the resin.

N'Hydroxybenzotriazole ester in N-methylpyrolidinone.
Symmetric anhydride in N-methylpyrolidinone.
Symmetric anhydride in DMF.
N'Hydroxybenzotriazole ester in DMF.

The isolated synthetic peptides were essentially homogeneous based on a highly loaded analytical HPLC (>95%) (Figs. 3 and 4), and they yielded satisfactory amino acid analyses (Table III).

Synthetic yields were relatively good. Based on amino acid analysis for unique residues incorporated near the end of the synthesis (glycine and histidine), the assembly of the fully protected peptide resins were approximately 60-5%. The data for the Val$^{33}$ analog indicated histidine at 60% and glycine at 64% of the loading of the internal norleucine standard. These results were corroborated by radioactivity measurements on peptide resin hydrolysates. The amount of $^{13}$C incorporated late in the synthesis was always 65% of norleucine, whereas the tritium label introduced early was recovered in the theoretical amount. The combined yield of the HF cleavage
The actual isolated yield of homogeneous analog obtained after conservative cuts of the HPLC fraction was about 10%; yields of the other analogs ranged up to 35%.

**Conformation of the Analogs**—The circular dichroism (CD) of solutions of glucagon and the synthetic analogs was measured as a function of solvent, concentration, pH, temperature, and time of standing in solution before measurement. The proportions of α helix, β sheet, and random coil were calculated as previously described (31) using the Prosec program, which is based on the conformational standards of Chang et al. (32). In a few instances, the results were confirmed by applying other programs to the data. Most of the measurements on glucagon were made on purified natural material, but in the several cases tested, synthetic glucagon was indistinguishable. All of the peptides were purified on preparative HPLC columns, lyophilized and stored in the freezer before dissolving in the 50% TFE/buffer and diluting to final composition and concentration. It has been shown (8-11) that glucagon aggregates in concentrated solution, and this effect is illustrated in Table IV. In 0.01 N HCl, the proportion of helix increased with concentration and in 0.01 M sodium phosphate, pH 9.2, containing 50% TFE, helicity decreased and β turns increased with concentration. The β turn values as determined by the Chang et al. (32) analysis can now be questioned, however. Since it has been established recently that there are at least three CD curves for various types of β turns (33-35), the values used as the basic spectra by Chang et al. (32) do not serve adequately in the deconvolution of CD curves. Under either condition the effect was minimal below 0.1 or 0.2 mg/ml and this concentration range was selected for further experiments. In vivo glucagon is found in the crystalline, helical structure in the storage granules, but it circulates in very dilute solution in the 10^-9-10^-10 M range, and is thought to exist as monomers with little secondary structure.

It is also well known that peptide conformation is strongly influenced by the presence of organic solvents in aqueous solutions and these conditions are thought to mimic the cell membrane where the hormone functions. Fig. 5 shows the mean residue ellipticity between 190 and 260 nm of [Glul32,Glu] glucagon in dilute solution at pH 6.9 (phosphate) in the presence of 10, 50, and 86% TFE. The shift in the shape of the CD curve is a clear indication of increased helicity and the calculations indicate changes from 5 to 50% in helix and 49 to 9% in β sheet as the organic solvent increases. These changes were rapid and reversible.

The effect of pH between 2 and 9.2 in dilute solutions of glucagon over the range of 0-86% TFE is illustrated in Fig. 6. The response was very similar at pH 2 and 9.2, with a sharp and parallel increase for α helix and decrease for β sheet at either pH extreme. At pH 6.9, the changes were qualitatively similar but more gradual. Addition of salt up to 0.2 M NaCl had no significant effect on the observed ellipticity. No significant differences were found between lyophilized samples measured within a few minutes after dissolution or after

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**Figure 4.** HPLC of crude and purified [Glul32,Glu] glucagon. Conditions: 0.4 x 30-cm μBondapak column; solvent A = 10% CH3CN in 0.1% aqueous H3PO4; solvent B = 50% CH3CN in 0.1% aqueous H3PO4, gradient 20-50% B in 60 min. Upper chromatogram, crude peptide after HF cleavage and dialysis; lower chromatogram, after purification as described in text.

**Table III**

| Amino acid | No. of residues | Val189 | [Glu20] | [Val189,Glu20] | [Glu18,Glul20] | [Ala18,Ala20] |
|------------|----------------|--------|---------|----------------|----------------|---------------|
| Asp        | 4              | 3.7    | 3.8     | 3.9             | 4.0            |
| Thr        | 3              | 2.7    | 2.6     | 2.4             | 2.9             | 2.5           |
| Ser        | 4              | 4.1    | 3.8     | 4.0             | 3.8             | 3.2           |
| Glu        | 3              | 3.1    | 4.0     | 4.2             | 5.0             |
| Gly        | 5              | 1.1    | 1.1     | 1.2             | 1.5             | 0.9           |
| Ala        | 0              | 0      | 0       | 1.2             | 1.2             | 3.0           |
| Val        | 0              | 0      | 0.9     | 0               | 0               |
| Met        |                | 0.9    | 0.9     | 0.9             | 1.0             |
| Leu        |                | 2.3    | 2.2     | 2.3             | 2.0             | 2.2           |
| Tyr        |                | 2.0    | 1.9     | 2.0             | 1.8             | 1.9           |
| Phe        |                | 1.0    | 1.1     | 1.2             | 1.0             | 0.9           |
| Lys        |                | 2.0    | 2.0     | 2.0             | 1.0             | 0.9           |
| His        |                | 1.0    | 1.0     | 1.0             | 0.9             | 0.8           |
| Arg        |                | 2.2    | 2.2     | 2.2             | 1.8             | 1.8           |
| Trp        |                | 1.0    | 1.0     | 1.0             | 0.6             |

*Hydrolyzed in 3 M p-toluenesulfonic acid.*

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and dialysis steps was 70 ± 5%, measured by analysis of glycine, histidine, and 14C in hydrolysates of the peptide resins and the cleaved and dialyzed peptides. Analytical HPLC indicated that the crude unpurified cleaved and dialyzed peptide mixture contained 65% of the desired target peptide, giving an overall synthetic yield of 60% x 70% x 65% = 27%. The actual isolated yield of homogeneous analog obtained...
the samules were warmed to 22 °C and read again. Chromatography on a CIS silica column with a 25-45% CH₃CN gradient. The CH₃CN was evaporated, and the aqueous solution was lyophilized. Samples and solvent composition. Commercial glucagon was freed of an impurity by reverse-phase chromatography on a C₄s silica column with a 25-45% CH₃CN gradient. The CH₃CN was evaporated, and the aqueous solution was lyophilized. Samples and solvent composition. Commercial glucagon was freed of an impurity by reverse-phase chromatography on a C₄s silica column with a 25-45% CH₃CN gradient. The CH₃CN was evaporated, and the aqueous solution was lyophilized.

Mean residue ellipticity of [Glu₂₂,Glu₂₃]glucagon as a function of trifluoroethanol concentration. 0.1 mg/ml in 0.01 M sodium phosphate, pH 6.9, 22 °C. a, 10% TFE; b, 50% TFE; c, 86% TFE.

Effect of pH on conformation of glucagon, 0.1 mg/ml.

Fig. 6. The conformation of glucagon as a function of pH and solvent composition.

Table V

Effect of time on conformation of dilute solutions of glucagon

Commercial glucagon was freed of an impurity by reverse-phase chromatography on a C₄s silica column with a 25-45% CH₃CN gradient. The CH₃CN was evaporated, and the aqueous solution was lyophilized. Samples of the solid were dissolved in 0.01 N HCl containing 0-90% TFE (final concentration, 0.14 mg/ml). CD readings at 22 °C were made within 30 min. After storage for 7 days at 4 °C, the samples were warmed to 22 °C and read again.

| TFE | α (Fresh) | α (7 days) | β (Fresh) | β (7 days) |
|-----|----------|-----------|----------|-----------|
| %   |          |           |          |           |
| 0   | 0        | 66        | 0        | 47        |
| 10  | 16       | 49        | 51       | 17        |
| 20  | 65       | 67        | 3        | 0         |
| 50  | 71       | 75        | 0        | 0         |
| 80  | 75       | 75        | 0        | 0         |

storage of the solutions at 0 °C for periods up to 7 days (Table V).

Conformational data on six of the synthetic glucagon analogs are shown in Table VI. Dilute solutions (0.1 mg/ml) in pH 6.9, 0.01 M phosphate, 22 °C, were examined as a function of TFE concentration. Peptide solubility limited the range to 10-86%. The analogs showed a narrower range of α or β conformation than glucagon itself. At low TFE (10%), [Val₉]⁴⁴ and [Glu₂²,Glu₂₃]glucagon were less helical than glucagon whereas [Val₉]⁴⁴, [Val₉,Glu₂₃]⁴⁴, [Glu₂₃]⁴⁴, and [Ala₂₂,Ala₂₃]glucagon were somewhat more. [Glu₂₃]glucagon was the most helical analog at 10% TFE; however, it changed slowly with increasing TFE and was the least helical at 86%. Similarly, it was lowest (49%) in β sheet at 10% TFE but changed very little with organic solvent, and it was still 42% at 86% TFE. [Val₉,Glu₂₃]glucagon behaved in much the same way. The analog expected to be most favorable for the β sheet structure in the 19-27 region was [Val₉]. However, it started with higher helicity than glucagon and remained relatively high (47%) even at 86% TFE. It began with somewhat less β structure, not more, than glucagon. The two analogs predicted to be strongly helical and low or negligible in β structure, [Ala₂₂,Ala₂₃]⁴⁴ and [Glu₂₃,Glu₂₃]⁴⁴ glucagon, did not show such a conformation in this system. They were actually less helical than glucagon at all concentrations of TFE and showed more β structure than the natural hormone at all TFE levels except at 10%.

Corresponding data on our analogs at acidic pH in 0.01 N HCl are shown in Table VII. In this case, the solubilities were sufficient to allow CD data to be measured at 0% TFE. In all analogs the helicity was very low in the aqueous solvent, but again rose with TFE concentration to values even higher than at pH 6.9. The β sheet was somewhat lower in 10% TFE than at pH 6.9 and dropped much more rapidly with rising TFE. The major difference seen at the two pH values were with analogs containing the additional glutamic acid residues at positions 22 or 23. At pH 6.9, [Glu₂₃]glucagon went from 12% helix in 10% TFE to only 26% helix in 86% TFE whereas, at pH 2, this analog rose from 0% helix at 0% TFE to 17% at 10% TFE and 71% at 86% TFE. [Val₉,Glu₂₃]⁴⁴ and [Glu₂₃,Glu₂₃]⁴⁴ glucagon behaved similarly. The acid medium suppresses the ionization of the γ carboxyl which, in turn, may promote the helical structure.

The helix of glucagon was also stabilized at lower temperatures (Fig. 7), although the effect was not dramatic. For example, at 5% TFE in pH 6.9 phosphate buffer, the apparent helix increased from 7 to 13% on going from 22 to −2 °C, an 86% increase. However, at 20% TFE, the change was from 48 to 52%, or only 8% increase. The proportion of β sheet decreased in a reciprocal manner and the calculated proportion of disordered, or random coil, peptide chain was essentially constant at all temperatures and solvent compositions.

The temperature effect also applied in 0.01 N HCl. The curves for α helix and β sheet at 4 °C are shown in Figs. 8 and 9 for glucagon and three of the synthetic position 22 and 23 analogs. The conformational increment between 22 and 4 °C was significant for glucagon, [Val₉]⁴⁴ glucagon, and [Ala₂₂,Ala₂₃]⁴⁴ glucagon, but was largest for [Glu₂₃,Glu₂₃]⁴⁴ glucagon.

In the experiments described here, a calculated value of 10% helix would mean that only 3 residues out of the 29 residues of glucagon would be in a helix if all molecules were in the same conformation, and that is not enough to stabilize such a structure. If the 19-27 segment of every molecule of glucagon changed from β sheet, or a random coil, to an α helix as a result of a solvent, temperature, or structural change, we would expect an increase from 0 to 9/29 = 31% helix. In 0.01 N HCl or pH 6.9 phosphate glucagon was 8% helix at 4 °C.
Addition of 13% TFE at 4 °C was needed (Fig. 8) to obtain the CD value equivalent to a full helix in the 19-27 segment, although the part of the molecule responsible for the helicity was not actually identified. This solvent may or may not correspond to the average polarity of a globular protein. The larger helicity detected at higher TFE means that other regions must also assume this structure.

Hormonal Activity of the Analogs—The purified glucagon analogs were assayed for biological activity by measuring the cAMP resulting from activation of adenylate cyclase in purified rat liver membranes. The response curves are shown in Fig. 10, and the results are in Table VIII. It can be seen that [Val22]glucagon gave a maximum response equivalent to that caused by excess glucagon in the production of cAMP, but more peptide was required. To reach a half-maximal response required 26 times as much peptide. The relative activity, therefore, was only 3.9%. [Tyr22]glucagon previously had also been shown to be a full agonist of 10% relative potency (31). [Val22]glucagon was 2.2% active but not a full agonist. None of the other analogs gave a full response in the concentrations that could be tested but [Val19, Glu23]-, [Glu23]-, and [Ala22, Ala23]glucagon were very weak partial agonists. [Glu22, Glu23]glucagon gave no detectable response even at 2 × 10⁻⁶ M and was less than 0.001% active. Since [Glu23]glucagon was such a weak partial agonist (0.1% relative potency), it could be demonstrated to be a competitive inhibitor of glucagon. A standard response curve of glucagon could be obtained in the presence of 5 × 10⁻⁷ M analog, where the analog alone gave no significant response. It was approximately parallel to the curve with glucagon alone but was shifted to the right. Thus 5.8 × 10⁻⁸ M glucagon was required.

**TABLE VI**

Conformation of glucagon analogs at neutral pH calculated from circular dichroism measurements

| Buffer | 0.01 M sodium phosphate, pH 6.9, 22 °C. Peptide concentration: 0.1 mg/ml. |
|---|---|

| Analog | Chou-Fasman predictions 19-27 segment | α helix | β sheet |
|---|---|---|---|
| | <P> | <P> | 10% TFE  | 20% TFE  | 50% TFE  | 70% TFE  | 80% TFE  | % | % |
| I Glucagon | 1.18 1.15 | 8 33 48 55 61 | 60 30 18 9 1.0 |
| II [Val19]glucagon | 1.14 1.25 | 11 28 42 47 | 54 33 19 15 |
| III [Val19]glucagon | 1.17 1.19 | 5 20 40 45 49 | 56 42 22 16 13 |
| V [Val19,Glu23]glucagon | 1.19 1.10 | 11 17 32 37 | 45 45 32 29 |
| VI [Glu23]glucagon | 1.23 1.00 | 12 14 23 26 | 49 50 43 42 |
| VII [Ala22,Ala23]glucagon | 1.25 0.99 | 9 19 38 44 50 | 49 44 25 18 11 |
| VIII [Glu22,Glu23]glucagon | 1.27 0.89 | 5 14 31 38 51 | 49 43 30 25 9 |

**TABLE VII**

Conformation of glucagon analogs at acidic pH calculated from circular dichroism measurements

| Solvent: 0.01 M HCl plus trifluoroethanol. Temperature: 22 °C. Peptide concentration: 0.1 mg/ml. |
|---|---|

| Analog | Chou-Fasman predictions | α helix | β sheet |
|---|---|---|---|
| | <P> | <P> | 0% TFE  | 10% TFE  | 20% TFE  | 50% TFE  | 80% TFE  | % | % |
| I Glucagon | 1.18 1.15 | 1 19 50 56 | 56 40 6 |
| II [Val19]glucagon | 1.14 1.25 | 2 13 38 56 | 55 44 25 6 6 |
| III [Val19]glucagon | 1.17 1.19 | 0 5 32 37 | 55 50 29 3 |
| V [Val19,Glu23]glucagon | 1.19 1.10 | 6 18 41 58 69 | 44 35 17 3 0 |
| VI [Glu23]glucagon | 1.23 1.00 | 0 17 42 65 71 | 75 35 16 0 0 |
| VII [Ala22,Ala23]glucagon | 1.25 0.99 | 0 8 30 37 | 55 47 24 |
| VII [Glu22,Glu23]glucagon | 1.27 0.89 | 0 13 42 55 | 55 43 14 |

**Fig. 7.** The conformation of glucagon as a function of temperature and solvent composition.

**Fig. 8.** Proportion of α helix in glucagon and position 22 and 23 analogs in 0.01 M HCl, 4 °C, as a function of trifluoroethanol concentration. Calculated with the Prosc program from circular dichroism data obtained on an Aviv 60DS CD spectropolarimeter. The concentrations of peptides were 0.1 mg/ml.
for half-maximal response compared with $6.9 \times 10^{-9}$ M in the absence of the inhibitor. The relative binding constants were approximately 9:1. Since this analog was a weak partial agonist, it could not be used in the concentration required for complete inhibition of glucagon and was not an effective antagonist.

The inhibition of glucagon by [Ala$^{22}$, Ala$^{23}$]glucagon was demonstrated by a different protocol (Fig. 11). In this experiment, a level of glucagon ($10^{-7}$ M) which alone gave 90% of maximal response was mixed in a series of tubes with increasing concentrations of analog. Total inhibition could be achieved at about $10^{-5}$ M and 50% inhibition was reached at $1.6 \times 10^{-6}$ M. The inhibition index, $I/A_0$, is the ratio of antagonist to agonist when the response was 50% of that due to agonist alone.

**FIG. 11.** The inhibition of glucagon by [Ala$^{22}$, Ala$^{23}$]glucagon in the adenylate cyclase assay. O, glucagon standard response curve; •, response to $10^{-7}$ M glucagon in the presence of increasing concentrations of [Ala$^{22}$, Ala$^{23}$]glucagon. $A$ is the concentration of agonist, $I$ is the concentration of antagonist. $I/A_0$ is the inhibition index, the ratio of antagonist to agonist when the response was 50% of that due to agonist alone.

**DISCUSSION**

The six new analogs of glucagon described here were prepared by solid-phase methods and purified to homogeneity. Aside from some chain termination at glutamine there were no special difficulties in syntheses. The composition and structure of the peptides were established, and we think they are of good quality and are suitable for the conformation and activity measurements undertaken.

The hypothesis we have been examining is that residue replacements in the 19-27 region of glucagon could be made which would cause predictable conformational changes observable by circular dichroism measurements. The predictions were based on the Chou-Fasman probability factors developed from examination of protein x-ray crystal structures (14). By increasing the $a$ helix or $\beta$ sheet potential of the replacements, several analogs were synthesized which were expected to cover a range of probabilities of being in one conformation or another and, in the extremes, were expected to cause the entire 9-residue 19-27 segment to change from a $\beta$ sheet to an $a$ helix or vice versa. Such a change should be easily detected by circular dichroism measurements on the whole 29-residue peptide. Percent changes from 0% $a$ and 51% $\beta$ to 31% $a$ and 20% $\beta$ were expected.

From the CD data, we find no clear correlation with the predicted $a$ helical and $\beta$ sheet potentials of the amino acids in this series of small 29-residue peptides. The predictions, of course, were derived from x-ray crystal structure data on globular proteins where the molecules are much larger and more rigid and have an inside and an outside which allows some residues to be exposed to solvent and some to be excluded from solvent. Even in solution such proteins maintain some structure. Glucagon has a largely helical structure in the crystal (7), but in solution it has much more conformational freedom and few if any buried residues. The structural flexi-
Conformation and Activity of Synthetic Glucagon Analogs

bility has been seen in NMR studies (12). It seems quite reasonable that the rules that govern the probabilities of individual amino acid residues being in a preferred conformation in a protein may not all apply to small peptides of this type. It has been shown previously that small polypeptides, e.g. preproparathyroid hormone (30 amino acid residues), can be predicted to have nearly equal probabilities of having two conformations (36). Under different environmental conditions, both predicted conformations have been verified by CD studies. In addition, based on the ellipticity at 210 nm, it has been concluded (37, 38) that the helicity of synthetic [Lys17, Glu21]glucagon was somewhat larger than that of glucagon itself in pH 9.2 phosphate. Fasman has reviewed this subject recently (39).

The positions of glucagon studied here were residues 19, 22, and 23. When Val22 or Phe22 and Val23 were replaced by a glutamic acid residue the potential for α helix in the 19-27 segment was markedly increased. However, these changes not only affected the predicted conformational potential but also the local charge, which may have a profound effect on structure. It was for this reason that the Ala22-Ala23 analog was selected because a Val replacement of Ala at 19 partly counteracted the high α potential increase due to replacement of Val22 by Glu23. Nonetheless, this analog was predicted to favor the helical conformation.

The second objective of this study was to try to correlate predicted and observed conformation in dilute solution with biological response. It was suggested (13) that the Ala17-Ala21 analog was also synthesized. In it the high helix potential was retained without a charge difference, although the hydrophobicity was less than the natural. The [Val19, Glu23]glucagon derivative was selected because a Val replacement of Ala at 19 partly counteracted the local charge, which may have a profound effect on structural conditions, both predicted conformations have been verified by CD studies. In addition, based on the ellipticity at 210 nm, it has been concluded (37, 38) that the helicity of synthetic [Lys17, Glu21]glucagon was somewhat larger than that of glucagon itself in pH 9.2 phosphate. Fasman has reviewed this subject recently (39).

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REFERENCES

1. Broner, W. W., Sinn, L. G., Staub, A., and Behrens, O. K. (1956) J. Am. Chem. Soc. 78, 3588-3589

2. Lund, P. K., Goodman, R. H., and Habener, J. F. (1981) J. Biol. Chem. 256, 6515-6518

3. Shields, D., Warren, T. G., Roth, S. E., and Brenner, M. J. (1981) Nature 289, 511-514

4. Kimball, C. P., and Murtin, J. R. (1923) J. Biol. Chem. 58, 337-346

5. Rodbell, M., Kraus, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1872-1876

6. Unger, R. H., and Orci, L. (1975) Lancet 14-16

7. Sasaki, K., Dockrell, A., Adamiak, D. A., Tickle, I. J., and Bundell, T. (1975) Nature 257, 751-757

8. Blanchard, M. H., and King, M. V. (1986) Biochem. Biophys. Res. Commun. 25, 298-303

9. Graeter, W. B., Beaver, G. H., Rattle, H. W. E., and Bradbery, E. M. (1968) Eur. J. Biochem. 3, 276-283

10. Serre, P. A., and Brooks, G. C. (1969) Arch. Biochem. Biophys. 129, 708-710

11. Moran, E. C., Chou, P. Y., and Fasman, G. D. (1977) Biochem. Biophys. Res. Commun. 77, 1300-1306

12. Boesch, C., Bungi, A., Oppliger, M., and Wüthrich, K. (1978) Eur. J. Biochem. 91, 209-214

13. Chou, P. Y., and Fasman, G. D. (1976) Biochemistry 14, 2536-2541

14. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 2, 45-148

15. Mitchell, A. R., Kent, S. B. H., Engelhard, M., and Merrifield, R. B. (1978) J. Org. Chem. 43, 2845-2852

16. Merrifield, R. B., Vizioli, L. D., and Boman, H. G. (1982) Biochemistry 21, 5000-5003

17. Moroder, L., Wackerle, L., and Wansch, E. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1647-1650

18. Andreu, D., and Merrifield, R. B. (1987) Eur. J. Biochem. 164, 585-590

19. Solomon, Y., Lodos, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548

20. Pohl, S. L. (1976) in Methods in Receptor Research (Blecher, M., ed) Part I, pp. 160-164, Marcel Dekker, New York

21. Markwell, M. A. K., Hass, S. M., Bieber, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206-210

22. Brown, R. L., Albano, J. D. M., Elkins, R. P., Sgherzi, A. M., and Tampon, W. (1971) Biochem. J. 121, 561-562

23. England, R. D., Jones, B. N., Flanders, K. C., Coolican, S. A., Rothgbe, T. M., and Gurd, R. S. (1982) Biochemistry 21, 940-950

24. Wright, D. E., and Rodbell, M. (1979) J. Biol. Chem. 254, 268-269

25. Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149-2154

26. Matsueda, G. R., and Gaede, S. A. (1979) in Peptides (Gross, E., and Meienhofer, J., eds) pp. 353-356, Pierce, Rockford, IL

27. Sarin, V., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) Anal. Biochem. 117, 147-157

28. Gian, B. F. (1972) Anal. Chem. Acta 58, 248-249

29. Mojsov, S., and Merrifield, R. B. (1984) Eur. J. Biochem. 145, 601-606

30. Tam, J. P., Heath, W. F., and Merrifield, R. B. (1983) J. Am. Chem. Soc. 105, 6442-6455

31. Lu, G. S., Mojsov, S., and Merrifield, R. B. (1987) Int. J. Pept. Protein Res. 29, 545-557

32. Chang, C. T., Wu, C.-S. C., and Yang, J. T. (1978) Anal. Biochem. 113, 31

33. Holois, M., Kawai, M., and Fasman, G. D. (1985) Biopolymers 24, 211-242

34. Holois, M., Kover, K. E., Holly, S., and Fasman, G. D. (1987) Biopolymers 26, 1555-1553

35. Holois, M., Kover, K. E., Holly, S., Radics, L., and Fasman, G. D. (1987) Biopolymers 26, 1555-1572

36. Rosenblatt, M., Beaudette, N., and Fasman, G. D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3983-3987

37. Kristensnky, J. L., Trivedi, D., Johnson, D., and Hruby, V. J. (1986) J. Am. Chem. Soc. 108, 1696-1698

38. Hruby, V. J., Kristensnky, J., Gynin, B., Telson, J. T., Trivedi, D., and Toon, R. L. (1986) Biopolymers 25, 5135-5155

39. Fasman, G. D. (1987) Biopolymers 26, S69-S79