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The Reaction of Glucagon with Its Receptor: Evidence for Discrete Regions of Activity and Binding in the Glucagon Molecule

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ABSTRACT Des-histidine-glucagon (DH-glucagon, glucagon$_{2-29}$) does not activate the glucagon-sensitive adenylate cyclase system present in either liver plasma membranes or in fat-cell "ghosts", but inhibits the response of these systems to submaximal concentrations of glucagon. DH-glucagon also inhibits, competitively, the binding of [^{125}I]glucagon to its receptor in liver plasma membranes. Amino-terminal fragments of glucagon (glucagon$_{1-21}$, glucagon$_{1-23}$) and carboxy-terminal fragments (glucagon$_{20-29}$, glucagon$_{22-29}$) failed to activate adenylate cyclase, to inhibit the response of the enzyme to glucagon, or to compete with labeled glucagon at its receptor.

It is concluded that the amino-terminal histidine residue of glucagon is essential for biological activity and that a hydrophobic near-carboxy-terminal region (residues 22–27) is essential for binding of glucagon to its receptor. Amino-terminal histidine may also contribute to the binding of glucagon, since the apparent affinity of DH-glucagon for the receptor is only about one-sixth that of glucagon. Thus, essentially the entire molecule of glucagon must be considered to be the biologically active species.

Because, as shown elsewhere, the binding of glucagon to its receptor shows characteristics of hydrophobic bonding, and because certain detergents induce conformational changes in the carboxy-terminal binding region of glucagon, the binding is probably of a lipophilic type.

Studies of the interactions between peptide hormones and their receptors help to clarify the relationship between structure and function in biological proteins. Interpretations with regard to hormone structure are feasible since many of the polypeptide hormones are single-chained, relatively low molecular weight compounds whose primary sequences are known. In addition, synthetic analogues with various degrees of biological activity are available for some of the hormones.

Since an analogue of a hormone may, for example, react with its receptor but not elicit a biological response, evaluation of structure–function relationships in peptide hormones requires two types of measurements: measurement of the binding between hormone or analogue and receptor, and measurement of the activity elicited by this reaction. For example, studies of the ability of various biologically active and inactive analogues of ACTH to displace labeled hormone from adrenal cortical cell membranes have revealed (1) that there are identifiable and distinct regions in ACTH that are necessary for binding and for biological activity.

We have shown (2) that glucagon, which consists of a single peptide chain of 29 amino acid residues, specifically activates an adenylate cyclase system in purified plasma membranes from rat liver. These membranes contain binding sites that are specific for glucagon and whose characteristics suggest that they are the receptors for this hormone (3). Fragments of glucagon (glucagon$_{1-21}$ and glucagon$_{22-29}$) did not activate adenylate cyclase and failed to compete with glucagon at its binding site, which suggests that most of the glucagon molecule is required for reaction with its receptor (3).

It has been reported recently (4, 5) that removal of amino-terminal histidine from glucagon results in loss of biological activity when this is tested either *in vivo* or *in vitro*. The present study shows that des-histidine-glucagon (DH-glucagon) competes with glucagon at its receptor site and inhibits the response of adenylate cyclase to glucagon. These findings, coupled with similar studies with other fragments of glucagon, have permitted conclusions to be drawn with regard to structure–function relationships in the glucagon molecule.

MATERIALS AND METHODS

Glucagon and glucagon$_{1-21}$ were supplied by Eli Lilly (Indianapolis); glucagon$_{1-21}$ contained less than 0.1% native glucagon as judged by adenylate cyclase assays. Glucagon$_{20-29}$ and glucagon$_{22-29}$ were obtained from Schering A.G. (Berlin) and from Dr. Victor Hruby (University of Arizona), respectively.

[^{125}I]Glucagon used in the binding studies was prepared and purified as described elsewhere (6). All reagents used for assaying adenylate cyclase activity have been described previously (7).

Preparation of DH-glucagon

The starting material was twice-crystallized porcine glucagon. The N-terminal histidine was removed by a one-step Edman degradation (8). After cleavage of the phenyl-thiocarbamylated glucagon with trifluoroacetic acid, the reaction mixture was added to water containing enough NaOH to bring the pH to approximately 10. The small amount of precipitate formed was removed by centrifugation. The pH of the supernatant liquid was brought to 5.3, and the mixture was allowed to stand at 4°C for 2 days. The precipitate formed consisted mainly of rhombododecahedral crystals. Electrophoresis of this material in 30% formic acid revealed a major and minor component that did not correspond to unmodified glucagon. DH-glucagon was purified from this mixture by isoelectric focusing over a linear pH gradient (5.5–9.0); the DH-glucagon peak appeared at pH 7.0. After the DH-glucagon fraction had

Abbreviation: DH-glucagon, des-histidine-glucagon.

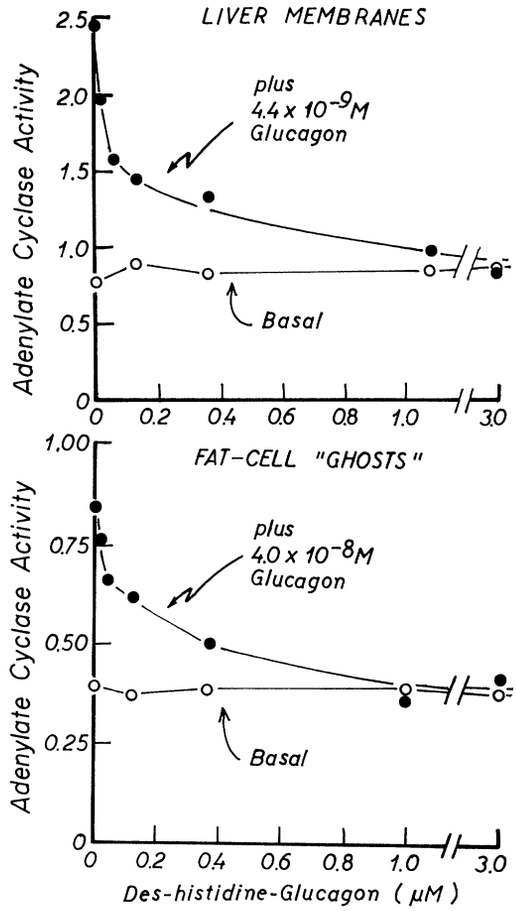

FIG. 1. Effects of des-histidine-glucagon on adenylate cyclase activity and its response to glucagon in rat liver plasma membranes and fat-cell "ghosts".

been desalted on a Sephadex G-25 column equilibrated with 0.1 N acetic acid, DH-glucagon was crystallized at pH 5.8 at 4°C. Comparison of the amino acid compositions of glucagon and the derivative (Table 1) revealed that the isolated derivative is DH-glucagon.

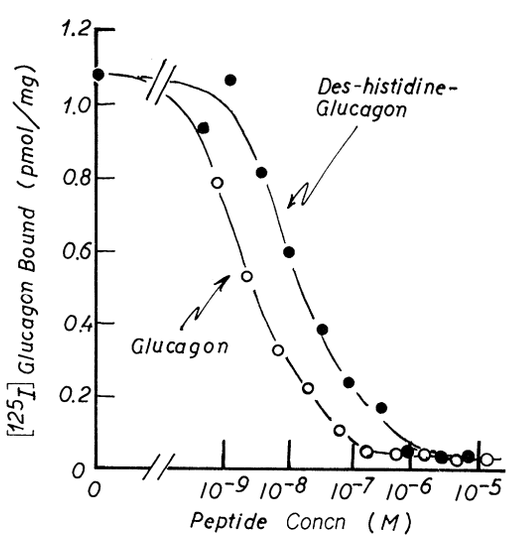

FIG. 2. Effect of des-histidine-glucagon and unlabeled glucagon on binding of [^{125}I]glucagon by liver membranes.

TABLE 1. *Total amino acid composition of glucagon and des-histidine-glucagon*

Amino acid	DH-glucagon Found*	DH-glucagon Theoretical†	Porcine glucagon Found*	Porcine glucagon Theoretical†
	(amino acid residues/mol)			
Try	— ‡	1	— ‡	1
Lys	0.88 (1)	1	0.96 (1)	1
His	0 (0)	0	0.96 (1)	1
Arg	1.94 (2)	2	1.91 (2)	2
Asp	3.98 (4)	4	4.17 (4)	4
Thr	2.89 (3)	3	2.75 (3)	3
Ser	3.64 (4)	4	3.43 (4)	4
Glu	3.12 (3)	3	3.29 (3)	3
Pro	0 (0)	0	0 (0)	0
Gly	1.05 (1)	1	1.35 (1)	1
Ala	1.03 (1)	1	1.18 (1)	1
Cys	0 (0)	0	0 (0)	0
Val	1.14 (1)	1	1.00 (1)	1
Met	0.96 (1)	1	1.07 (1)	1
Ile	0 (0)	0	0 (0)	0
Leu	2.00 (2)	2	2.00 (2)	2
Tyr	1.82 (2)	2	2.03 (2)	2
Phe	1.96 (2)	2	1.99 (2)	2
Total	(27)	28	(28)	29

* Figures in parentheses are the nearest integer.
† According to Bromer *et al.* (25).
‡ Not determined. Specific absorption at 280 nm indicated complete recovery of tryptophan and tyrosine in DH-glucagon.

Preparation of liver plasma membranes and fat-cell "ghosts"

Partially purified plasma membranes from rat liver were prepared by a modification (9) of the method of Neville (10). Fat-cell "ghosts" were prepared from isolated fat cells of rat adipose tissue as described elsewhere (11).

Adenylate cyclase assay

Liver plasma membranes (0.5 mg protein/ml) or fat-cell ghosts (0.86 mg protein/ml) were incubated in medium containing 3.2 mM [α-^{32}P]ATP (35 cpm/pmol), 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM 3′:5′-cyclic AMP (cAMP), 20 mM creatine phosphate, 1 mg/ml of creatine kinase, and 25 mM Tris·HCl, pH 7.6. Incubations were for 10 min at 30°C. The quantity of cAMP formed was measured by the procedure of Krishna *et al.* (12). Adenylate cyclase activities are expressed as nanomoles of cAMP formed in 10 min per mg of protein.

Binding assay

Liver plasma membranes (0.2 mg protein/ml) were incubated at 30°C in medium containing 2.5% bovine serum albumin, 20 mM Tris·HCl, pH 7.6, and 1.5×10^{-9} M [^{125}I]glucagon (6×10^5 cpm/pmol), with or without the indicated concentrations (see Fig. 2) of unlabeled glucagon or various peptide fragments of the hormone. Incubation time was 15 min. Membranes were sedimented, washed, and counted as described elsewhere (3). Results are expressed as picomoles of glucagon bound per mg of membrane protein.

RESULTS AND DISCUSSION

Experiments *in vivo* have shown that DH-glucagon has neither glycogenolytic nor insulinogenic effects. DH-glucagon also

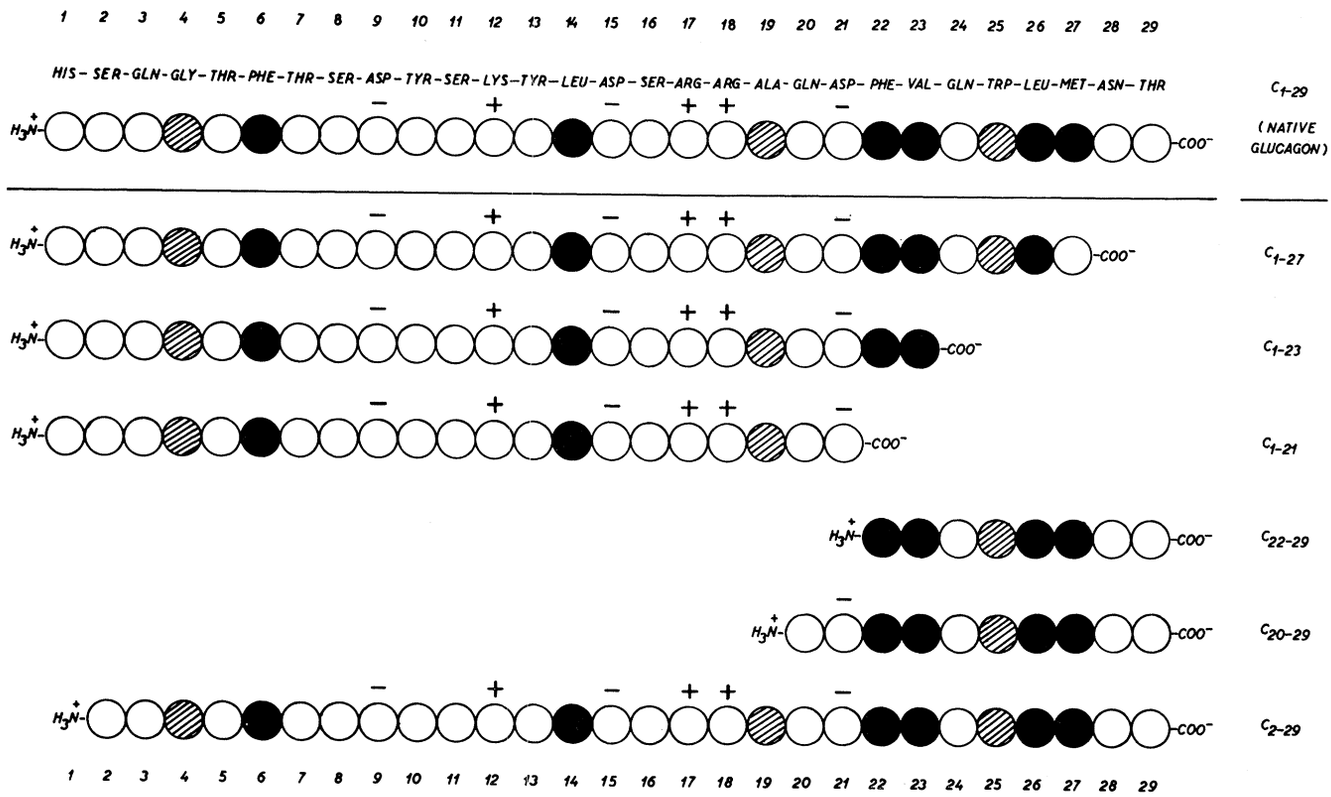

FIG. 3. Polar and hydrophobic characteristics of amino acids in native glucagon and glucagon fragments whose properties are summarized in Table 2. Polar residues are indicated by open circles, nonpolar residues by closed circles, and residues with intermediate polarity by hatched circles. Residues that are positively or negatively charged at pH 7.4 are indicated by + or −, respectively.

The classification of amino acids according to their polarity is that of Dickerson and Geis (24) and was chosen to emphasize the fact that a region with a high concentration of unequivocally hydrophobic amino acids is present near the carboxy terminus of glucagon. The extremely low solubility in water of the 22–29 and 20–29 peptide fragments supports this classification. Certain amino acids that are designated here as polar, notably Tyr, Gln, and Asn, interact with surrounding structures primarily by hydrogen bonds, hydrophobic bonds, or a combination of the two depending on the microenvionomment in which they are located. Replacementof open circles by closed or hatched circles for these residues does not change the conclusion regarding the relative hydrophobicity of theregion near the carboxy terminus.

has no insulin-releasing or lipolytic effects when incubated with isolated rat islets and fat cells, respectively (4, 5). Since glucagon stimulates glycogenolysis (13), lipolysis (14), and release of insulin (15) through its action on adenylate cyclase systems, we examined the effects of DH-glucagon on adenylate cyclase activity in fat-cell "ghosts" and in partially purified plasma membranes from liver.

Consistent with its failure to exhibit biological activity *in vivo* or with glucagon-responding cells or tissues, DH-glucagon had no effect, even at high concentrations, on the activity of the adenylate cyclase systems of liver membranes and fat-cell "ghosts" (Fig. 1). However, DH-glucagon inhibited, in both systems, the response of adenylate cyclase to glucagon. These findings suggested that although DH-glucagon is biologically inactive, it may compete with glucagon at the receptor. This possibility was tested directly by comparing the effects of DH-glucagon and of unlabeled native glucagon on the binding of [^{125}I]glucagon to its receptor in liver plasma membranes. As illustrated in Fig. 2, DH-glucagon inhibited, competitively, the binding of labeled glucagon. The apparent affinity of DH-glucagon for the receptor is approximately one-sixth that of glucagon.

In experiments of the type described in Figs. 1 and 2, it was found that fragments of glucagon consisting of residues

TABLE 2. *Biological activity and binding properties of glucagon fragments*

	Properties		
	Adenylate cyclase assay		Binding assay
Fragment	Stimulation of activity	Inhibition of glucagon stimulation	Competition with glucagon at receptor
1–27	+	NT	NT
1–23	−	−	NT
1–21	−	−	−
22–29	−	−	−
20–29	−	−	−
2–29	−	+	+

NT, not tested.

1–21, 20–29, and 22–29, when added at concentrations of about 10^{-7} M, did not stimulate adenylate cyclase and failed to inhibit either the effects of glucagon on adenylate cyclase or the binding of labeled glucagon to liver membranes. In Fig. 3 and Table 2 are summarized the present findings, plus those reported by Spiegel and Bitensky (16) for the effects of glucagon$_{1-23}$ and glucagon$_{1-27}$ (in which methionine at resi-

due 27 is replaced by homoserine) on hepatic adenylate cyclase activity.

Two main points emerge from these findings: (*a*) N-terminal histidine is essential for biological activity of glucagon; deletion of this residue results in loss of biological activity but retention of the ability of the remainder of the molecule to bind with receptor; and (*b*) The carboxy-terminal region occupied by amino acid residues 22–27 seems to be essential for binding of glucagon to its receptor (glucagon$_{1-27}$, though not tested for binding, must bind since it is biologically active; glucagon$_{1-21}$ has been shown directly not to compete with glucagon at the receptor, and glucagon$_{1-23}$ presumably does not compete since it does not inhibit glucagon stimulation). Since neither of the carboxy-terminal fragments 20–29 and 22–29 competes with glucagon at the receptor, additional interacting residues must exist. One of the additional binding sites may be N-terminal histidine since DH-glucagon has a lower apparent affinity for the receptor than does glucagon. We are currently investigating whether blockage of free amino groups in glucagon (N-terminal histidine, Lys-12) and modifications of Arg-Arg residues at positions 17 and 18, a region of high charge density, may alter binding of glucagon to its receptor.

Since essential regions for function and for binding include both the amino-terminal and the (22–27) near-carboxy-terminal regions of glucagon, it is apparent that the entire molecule represents the biologically active species. Thus, further understanding of the relationships in the glucagon molecule between structure, function, and binding should be derived mainly from studies of its conformation (or stereochemistry) in the receptor environment. Since the glucagon receptor has not been isolated, such studies are not possible at this time. However, recent studies on the properties of the receptor in liver membranes (3) and studies of conformational changes of glucagon using detergents (17) as a possible model for the lipophilic qualities of membranes (or receptors) may provide clues to the stereochemical possibilities involved in glucagon-receptor interactions.

In dilute aqueous solution, glucagon is essentially a structureless polypeptide chain (18, 19). The only evidence of intramolecular interactions stems from weak circular dichroic activity in the 220 nm region. If the dichroic activity is derived exclusively from the peptide chromophore, it represents about one turn of an α-helix. It has been found recently (17) that glucagon "binds" cetyl trimethylammonium bromide and in the process acquires tertiary structure, as evidenced by changes in various properties of the tryptophan chromophore. The latter is in the hydrophobic, carboxy-terminal portion of glucagon (Fig. 3). These findings indicate that the environment of the molecule can have an important influence on the folding of glucagon. It is perhaps significant that the hydrophobic, near-carboxy-terminal region exhibits strong interactions with detergents and is an essential region of binding of glucagon at its receptor.

The receptor for glucagon is destroyed by treatment of fat cells with trypsin (20), which suggests that the receptor contains a protein. It has also been found that treatment of liver membranes with phospholipases or digitonin abolishes both the binding of glucagon and hormonal activation of adenylate cyclase (3, 7). Addition of phospholipids to the treated membranes partially restores both processes (3). It seems, therefore, that lipids contribute to the structural properties of the receptor.

It has also been found (7) that binding of glucagon to liver membranes is markedly reduced either as the incubation temperature is lowered or when the membranes are incubated with low (0.4 M) concentrations of urea. Decreased stability with reduced temperature or with the addition of urea are characteristic of hydrophobic interactions (21, 22) and this provides evidence, therefore, that such interactions are important for the binding of glucagon to its receptor. Coupled with the model studies cited above, these findings suggest that receptor lipids may participate in binding and induction of conformational changes of the carboxy-terminal hydrophobic region of glucagon at the receptor.

The finding that N-terminal histidine is essential for the biological activity of glucagon should prove valuable for determining the nature of the reaction induced by glucagon at its receptor. Further studies will be required to determine whether other amino acid residues in the amino-terminal region are also involved in the functional properties of glucagon. In this regard, it is of great interest that secretin, a peptide hormone of the duodenal mucosa, also has an N-terminal histidine residue and has amino acid residues homologous to those of glucagon in the amino-terminal region (residues 1–7) (23). Secretin and glucagon activate the same adenylate cyclase system in fat cells of the rat, but not through the same receptors (20). Secretin also does not compete with glucagon or act on the glucagon-sensitive adenylate cyclase system of liver membranes (3, 9, 16). Since these hormones have virtually identical amino-terminal (or functional) regions and different receptors, it is reasonable to suggest that the mechanism of action of the two hormones on their respective receptors is identical, but that specificity of binding depends on portions of the molecules other than the amino-terminal region. Comparative studies of the binding and functional components of secretin at its receptor and manipulations of the amino acid sequence in secretin and glucagon seem appropriate for future studies of function–structure relationships in these hormones.

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