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Structure–Function Relationships in Glucagon: Properties of Highly Purified Des-His[1]-, Monoiodo-, and [Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon†

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ABSTRACT: We have compared the ability of glucagon and three highly purified derivatives of the hormone to activate hepatic adenylate cyclase (an expression of biological activity of the hormone) and to compete with [^{125}I]glucagon for binding to sites specific for glucagon in hepatic plasma membranes. Relative to that of glucagon, biological activity and affinity of [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon, prepared by CNBr treatment of glucagon, were reduced equally by 40- to 50-fold. By contrast, des-His[1]-glucagon, prepared by an insoluble Edman reagent and highly purified (less than 0.5% contamination with native glucagon), displayed a 15-fold decrease in affinity but a 50-fold decrease in biological activity relative to that of the native hormone. At maximal stimulating concentrations, des-His[1]-glucagon yielded 70% of the activity given by saturating concentrations of glucagon. Thus, des-His[1]-glucagon can be classified as a partial, weak agonist. Highly purified monoiodoglucagon and native glucagon displayed identical biological activity and affinity for the binding sites. Our findings suggest that the hydrophilic residues at the terminus of the carboxy region of glucagon are involved in the process of recognition at the glucagon receptor but do not participate in the sequence of events leading to activation of adenylate cyclase. The amino-terminal histidyl residue in glucagon plays an important but not obligatory role in the expression of hormone action and contributes to a significant extent in the recognition process.

The first event in the cascade of reactions leading to hormone response is the interaction of the hormone with its recognition site termed the "receptor". It is commonly assumed that binding of the hormone to the receptor induces certain transformations in the responding system. Studies of the structural requirements for hormonal recognition and action should provide further understanding of the mechanism of hormone action.

Although the structure of glucagon has been known since 1957 (Bromer et al., 1957), the structure–function relationships for this polypeptide hormone have not been elucidated clearly as yet. The discovery of a primary target for glucagon, namely the adenylate cyclase system (for review, see Rodbell, 1972; Sutherland, 1972), has provided a means of evaluating these relationships. Numerous studies have been reported with chemically modified derivatives of glucagon in attempting to establish the structure–functional role of each amino acid residue in the peptide (Spiegel and Bitensky, 1969; Rodbell et al., 1971a; Grande et al., 1972; Lande et al., 1972; Epand and Epand, 1972; Epand, 1972; Epand et al., 1973). One major obstacle in obtaining clear-cut results is the purity of the various glucagon derivatives. Chemical modification seldom produces complete conversion of substrate to product. Therefore, extensive purification is essential to remove any remaining native glucagon. Characterization of each derivative requires the study of its affinity for the receptor as well as its intrinsic activity in stimulating adenylate cyclase activity. Obviously, a significant level of contaminating glucagon would make interpretation difficult.

[Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon (CNBr-glucagon),[1] prepared by cyanogen bromide cleavage of glucagon, has been examined in two studies (Spiegel and Bitensky, 1969; Epand, 1972). In both cases, attempts to separate glucagon from CNBr-glucagon by either gel filtration or gel electrophoresis proved unsuccessful. Unreact-

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[1] Abbreviations used are: CNBr-glucagon, [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon; DH-glucagon, des-His[1]-glucagon; cyclic AMP, 3′,5′-adenosine monophosphate.

ed glucagon was estimated by amino acid analysis to be 2 to 3% of the final product. Thus, while CNBr-glucagon showed reduced activities relative to that of glucagon, it is not known whether this derivative has the same maximal activity as the native hormone. There is also no information available on its affinity for the glucagon receptor.

[^{125}I]Glucagon is commonly used in studies of hormone binding. In the first such study of glucagon binding (Rodbell et al., 1971b), monoiodoglucagon was shown to have an identical concentration–activity dependency as native glucagon on hepatic adenylate cyclase. A recent study (Bromer et al., 1973), however, suggested that monoiodoglucagon has a greater activity in stimulating hepatic adenylate cyclase. If various iodinated derivatives indeed have different potencies from the native hormone, then the interpretation of the binding of [^{125}I]glucagon to membranes containing the glucagon receptor would be difficult.

Des-His^1-glucagon (DH-glucagon), prepared by conventional Edman one-step degradation (Rodbell et al., 1971a), showed a lack of biological activity on the hepatic adenylate cyclase system although the derivative binds to specific binding sites for glucagon in hepatic plasma membranes with about one-tenth the affinity of native glucagon. DH-glucagon prepared by Lande et al. (1972), judged by them to contain about 6% glucagon, apparently lacked the ability to activate adenylate cyclase; their studies also indicated that N^{ϵ}-phenylthiocarbamylglucagon, a potential product of glucagon formed by the Edman procedure, is likely to be biologically inactive.

In this study we have prepared CNBr-glucagon and purified this material to the extent that it contains less than 0.5% native glucagon. DH-Glucagon has been prepared by the insoluble Edman reagent described by Dowling and Stark (1969). In contrast to the conventional method, this procedure allows complete separation from the lysine-substituted derivative. Although the yields of DH-glucagon obtained by this procedure remain to be improved, it is feasible to prepare large quantities containing less than 0.5% glucagon. The glucagon-sensitive hepatic adenylate cyclase system (Pohl et al., 1971) is used for assaying biological activity of monoiodoglucagon, CNBr-glucagon, and DH-glucagon. The affinities of these derivatives for specific glucagon binding sites in hepatic plasma membranes (Rodbell et al., 1971b) have been compared with that of native glucagon as a means of determining the structural requirements for recognition vs. action of the derivatives and the native hormone.

Experimental Section

Materials. [α-^{32}P]ATP was obtained from International Chemical and Nuclear; [^{3}H]cAMP was from New England Nuclear. ATP, cyclic AMP, creatine phosphate, creatine phosphokinase, and dithiothreitol were purchased from Sigma. CNBr was from Eastman Kodak. Oxoid membranes were purchased from Amersham–Searle. Chemicals used in the solid phase Edman procedure were Sequanal grade from Pierce or best grade available and were used without further purification. Polystyrene beads crosslinked with 0.25% divinylbenzene (no. L982) (from Sondell Scientific Instruments, Palo Alto, Calif.) were a gift from Dr. George R. Stark. Crystalline porcine glucagon was provided by Eli Lilly and Co. or was purchased from Schwarz/Mann; purified monoiodoglucagon was a gift from Dr. W. W. Bromer of Eli Lilly. Solvents for partition chromatography were purified as previously described (Hruby and Groginksy, 1971).

Preparation of Plasma Membranes from Rat Liver. Partially purified plasma membranes from rat liver were prepared by a modification of the procedure of Neville (1968) as previously described (Pohl et al., 1971), and stored in liquid nitrogen.

Assay for Adenylate Cyclase Activity. The assay medium consisted of [α-^{32}P]ATP (about 40 cpm/pmol) at 2 m*M*; $MgCl_2$, 5 m*M*; cyclic AMP, 0.4 m*M*; dithiothreitol, 1 m*M*; bovine serum albumin, 2 mg/ml; creatine phosphate, 5 m*M*; creatine phosphokinase, 0.3 mg (50 units)/ml; and Tris-Cl buffer, 30 m*M* at pH 7.5 in a final volume of 100 μl. Liver membranes, suspended in Tris-Cl buffer containing 1 m*M* dithiothreitol, were added to a final concentration of 0.2–0.5 mg/ml to initiate the reaction. After 5 min at 30°, the reaction was terminated by adding 100 μl of stopping solution (Salomon et al., 1974). Cyclic AMP formed was determined by a recently developed procedure with the use of Dowex 50 and alumina columns (Salomon et al., 1974).

Assay for [^{125}I]Glucagon Binding. [^{125}I]Glucagon was prepared as described previously (Rodbell et al., 1971b). The concentration of biologically active glucagon in the preparations of labeled hormone was estimated from assays of adenylate cyclase activity (see above); activities were compared with those generated by native glucagon over a wide range of concentrations. The incubation medium used for the binding assay contained [^{125}I]glucagon at $1–5 \times 10^{-9}$ *M* (see legend for specific activity); bovine serum albumin, 3 mg/ml; liver membrane, 50–100 μg/ml; and Tris-Cl buffer, 20 m*M* at pH 7.5 or as indicated in a final volume of 1 ml. The assay was initiated by the addition of liver membranes and the whole mixture was poured on an oxoid membrane filter after 10 min at 30°. The filter was quickly washed twice with 1 ml of cold Tris-Cl buffer containing 2 mg/ml of bovine serum albumin. Suction was applied during the washing so that each wash took less than 5 sec to complete. Then the filter was counted in a well-type Packard γ-counter.

Analytical Methods. Protein concentration was determined according to Lowry et al. (1951) with serum albumin as standard. The amino acid content was analyzed, after the hydrolysis with methanesulfonic acid (Liu, 1972), on a JEOL JLC-6AM analyzer. No correction is made for destruction of amino acids during hydrolysis. Glucagon concentration was determined by its absorbance at 280 nm (molar absorbance 8050) or by amino acid analysis. Fresh glucagon solution (in 0.1% bovine serum albumin and 20 m*M* Tris-Cl buffer, pH 7.5) was prepared each time prior to use, since we have found that storage of glucagon in solution decreased its effectiveness in activating adenylate cyclase.

Results

Preparation of DH-Glucagon. When DH-glucagon is prepared by the conventional one-step Edman degradation (Rodbell et al., 1971a), the difference between the pK_a values of the α- and ϵ-amino groups is too small to limit the phenyl isothiocyanate reaction to only the α-terminal histidyl residue. Therefore, the product requires extensive purification. It occurred to us that the insoluble Edman reagent (Dowling and Stark, 1969), where products of the reaction of the side-chain ϵ-amino groups are not recovered, might

provide an ideal procedure for the preparation of DH-glucagon. The procedure for preparing glucosaminol-isothiocyanate-polystyrene described by Dowling and Stark (1969) was followed closely. After the reagent was prepared, a column (1 × 15 cm) of the isothiocyanate resin was packed, thoroughly washed with pyridine buffer (pyridine–*N*-ethylmorpholine–H_2O (15:1.4), adjusted to pH 8.1 with glacial acetic acid), and maintained at 55° for the following reactions. Glucagon, 15 μmol, was dissolved in 2.5 ml of the same pyridine buffer and about 5×10^6 cpm of [^{125}I]glucagon was added to monitor the recovery. After the sample was placed on the resin, 1 ml of the buffer was added to wash down the sample. All solvents used subsequent to the coupling step contained 1% mercaptoethanol to protect against oxidation. After allowing coupling of glucagon through its amino groups to the resin to proceed for 1.5 hr at 55°, the column was washed twice with 25 ml of pyridine buffer and six times with 25 ml of pyridine, until no radioactivity was detected in the eluate. The resin was washed again with four 50-ml portions of tetrahydrofuran, and the tetrahydrofuran was displaced by adding 3 ml of the cleavage solvent (trifluoroacetic acid–glacial acetic acid (4:1)) to the column; the column flow was stopped and 10 ml of cleavage solvent was added. After 30 min with occasional stirring, this step was repeated once to ensure a good yield of cleavage product. Finally the column was washed with 8 ml of the same solvent. All the cleavage eluates were combined and lyophilized to dryness. The yield of the DH-glucagon at this stage ranged from 10 to 20%. The product obtained at this stage has a brownish color; its amino acid analysis showed the presence of glucosamine and about 0.15 residue of histidine. Since the resin contained covalently bound glucosaminol and after extensive washing the only source for free histidine was the glucagon coupled through ϵ-NH_2 groups to the resin, it appears that hydrolysis had occurred at the isothiocyanate group and thus glucosaminol and intact glucagon were released. This contaminant was removed by chromatography on a Dowex 50 column. The product was dissolved in 5 ml of 0.1 *M* pyridine–acetate buffer (pH 3.3) and applied to a AG50W-X2 column (0.9 × 9 cm), equilibrated with the same buffer at 55°. After washing the column with 20 ml of 0.77 *M* pyridine–acetate buffer (pH 4.4), DH-glucagon was eluted with 60 ml of 1.38 *M* pyridine–acetate buffer (pH 4.8). After being concentrated to 3 ml, the product was gel filtered on a Sephadex G-10 column (1.5 × 28 cm) with 10% acetic acid and then lyophilized. The yield was 5–10%.

In order to ascertain the amount of unreacted glucagon in the final product, a large amount (20 nmol) of peptide was applied to the amino acid analyzer after methanesulfonic acid hydrolysis. With suitable controls, it was established that the analyzer could detect histidine at levels of 0.1 nmol as a distinct peak. As shown in Table I, DH-glucagon contained less than 0.5% of histidine residue whereas all other amino acid residues, including tryptophan and lysine, were unchanged. We conclude from this analysis that DH-glucagon contains at most 0.5% contaminating glucagon.

Preparation of CNBr-Glucagon. The preparation of CNBr-glucagon followed that general method for cyanogen bromide cleavages of peptides and proteins (Steers et al., 1965). Glucagon (12.0 mg or 3.70 μmol) was dissolved in 12 ml of 70% formic acid (redistilled), and 110 mg of cyanogen bromide was added. The mixture was stirred for 14 hr at 25° and then 30 mg more of cyanogen bromide was added. After a total of 24 hr the solution was lyophilized.

Table I: Amino Acid Compositions of Glucagon and Its Derivatives.[a]

Amino Acids	Porcine Glucagon		DH-Glucagon		CNBr-Glucagon	
	Found	Theory	Found	Theory	Found	Theory
Trp	0.98	1	0.93	1	0.88	1
Lys	1.03	1	1.08	1	1.00	1
His[b]	0.88	1	<0.005	0	0.82	1
Arg	2.00	2	1.83	2	2.00	2
Asp	3.93	4	4.29	4	2.68	3
Thr	2.88	3	2.51	3	1.73	2
Ser	3.46	4	3.38	4	3.60	4
Glu	3.03	3	3.28	3	3.03	3
Gly	0.95	1	1.10	1	1.07	1
Ala	0.93	1	1.03	1	0.93	1
Val	0.89	1	0.90	1	0.86	1
Met[b]	0.93	1	1.07	1	<0.005	0
Leu	2.05	2	2.23	2	2.02	2
Tyr	1.92	2	1.84	2	2.02	2
Phe	2.00	2	1.84	2	2.02	2

[a] Amino acid compositions were analyzed after 20-hr hydrolysis at 115° in 4 *N* methanesulfonic acid containg 0.2% tryptamine. The results expressed in residues per molecule are uncorrected for any destruction during hydrolysis. [b] Histidine and methionine were measured in separate analyses, in which 20 or 30 nmol of DH- or CNBr-glucagon, respectively, was applied. In either analysis, no distinct peak was detected; it was established that the analyzer could detect 0.1 nmol of amino acid as a distinct peak.

A clean separation of native glucagon (R_F 0.32) from CNBr-glucagon (R_F 0.57) was obtained by slightly modifying a solvent system already developed for partition chromatography of glucagon (Hruby and Groginsky, 1971). The system used was 1-butanol–ethanol–benzene–0.2 *N* ammonium hydroxide (5:2:1:8) adjusted to pH 9.4 by acetic acid. The sample was dissolved initially in 1 ml of 50% acetic acid, and then 2 ml of upper phase was added. The mixture was placed on a 2.2 × 57 cm column of Sephandex G-25 (100–200 mesh) which had been equilibrated with the upper and lower phases (Hruby and Groginsky, 1971). The peptide peak was determined using the method of Lowry et al. (1951); the CNBr-glucagon peak was pooled and lyophilized. The peptide was subjected to gel filtration on Sephadex G-15 (1 × 56 cm) using 50% acetic acid. After lyophilization, 3.4 mg of white powder was obtained for a yield of 31%.

Amino acid analysis showed no methionine (<0.5%, Table I) even when the concentration of the analyzed sample was extremely high.

Activation of Hepatic Adenylate Cyclase by Glucagon Derivatives. A typical concentration–activity curve for native glucagon is shown in Figure 1. Activation of adenylate cyclase was detectable at 2×10^{-10} *M* glucagon and maximal activity was reached at 4×10^{-8} *M* glucagon. By contrast, DH-glucagon did not stimulate adenylate cyclase activity until its concentration reached 3×10^{-9} *M*. Furthermore, even at 10^{-6} *M*, a maximal stimulating concentration, DH-glucagon stimulated less than 70% of the maximal activity engendered with native glucagon. Based on the concentration of DH-glucagon required to give activities equivalent to that given with glucagon, it was estimated that the derivative, on a molar basis, has about 2% of the intrinsic activity given by native glucagon. Judging from amino acid analysis (Table I) and the nature of the concentration–ac-

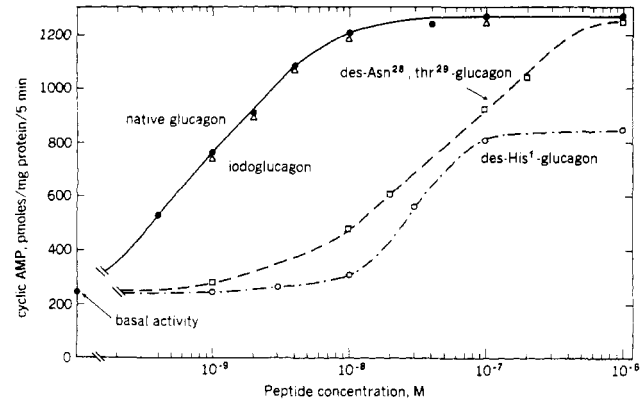

FIGURE 1: Concentration-activity curves for porcine glucagon and its derivatives. The activity of hepatic adenylate cyclase was measured as described in the Experimental Section. The concentration of each peptide was estimated from its absorbance at 280 nm or by amino acid analysis.

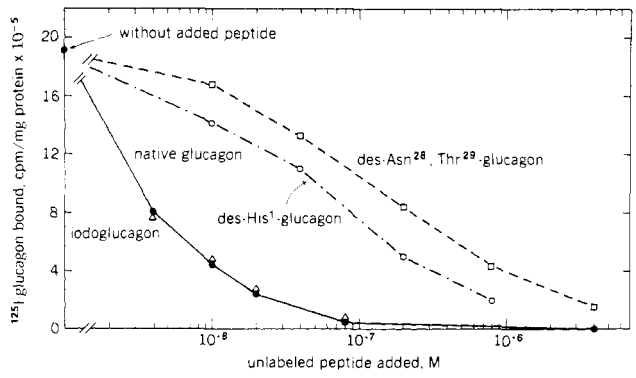

FIGURE 2: Effect of addition of unlabeled peptides on the binding of [^{125}I].glucagon. The binding assays were carried out with 4×10^{-9} *M* [^{125}I]glucagon (specific activity, 10^6 cpm/pmol) as described in the Experimental Section. Unlabeled peptides were added at the concentrations indicated.

tivity curve, it is highly unlikely that the activity given by DH-glucagon is due to contaminating glucagon. Thus, while confirming a previous report (Rodbell et al., 1971a) that the NH_2-terminal histidine plays an important role in the expression of hormone action, our data show that this residue is not essential for hormone action as previously thought.

In addition to removal of the terminal threonine and asparagine residues of glucagon, CNBr treatment converts methionine to homoserine lactone. These changes in the structure of glucagon reduced its effectiveness to activate the adenylate cyclase system by 40- to 50-fold (Figure 1). However, CNBr-glucagon, in contrast to DH-glucagon, was capable of causing the same degree of activation of adenylate cyclase as the native hormone. Judging from the amino acid analysis (Table I), contaminating glucagon was less than 0.5%; therefore, the activity observed must represent the intrinsic activity of CNBr-glucagon.

It has been reported that the incorporation of iodine into the tyrosyl residues of glucagon reduces the concentration necessary for half-maximal stimulation of adenylate cyclase activity by about fivefold (Bromer et al., 1973). Previously it had been reported (Rodbell et al., 1971b) that glucagon iodinated with I_2 was essentially identical with glucagon in its ability to stimulate adenylate cyclase activity. Because of the widespread use of iodinated glucagon in binding and activity studies, we considered it important to reexamine the effects of monoiodoglucagon, kindly supplied by Dr. Bromer, on the adenylate cyclase system. As shown in Figure 1, native and monoiodoglucagon gave essentially the same concentration-activity curves. As shown below (Figure 2), the relative affinities of glucagon and monoiodoglucagon for the glucagon binding sites in hepatic membranes are also the same.

Glucagon Binding and Competition with Its Derivatives. Hepatic plasma membranes contain binding sites that are highly specific for glucagon (Rodbell et al., 1971b). The relative affinities of native glucagon and the various derivatives for these sites were determined by their ability to compete with the binding of [^{125}I]glucagon to hepatic membranes. As judged by the concentrations required for 50% displacement of the labeled hormone, monoiodoglucagon has the same affinity as native glucagon for the binding sites whereas the affinities of DH-glucagon and CNBr-glucagon for these sites were shifted 15-fold and 50-fold, respectively (Figure 2). It should be emphasized that the loss of affinity of the modified glucagon derivative lacking only histidine was relatively small (15-fold) (in three other experiments of this type, the average was 17-fold) when compared to its loss of ability to activate the adenylate cyclase system (greater than 50-fold). On the other hand, CNBr-glucagon displayed nearly identical decreases in both affinity for the binding sites and in ability to activate the adenylate cyclase system (40- to 50-fold). It is reasonable to conclude from these data that modification at the COOH-terminal region of glucagon by treatment with cyanogen bromide significantly reduces the affinity of the peptide for its receptor but apparently does not affect the intrinsic activity of the hormone derivative as compared to the native hormone. On the other hand, modification of the amino-terminal region of the hormone by removal of the terminal histidyl group not only significantly reduced the affinity of the peptide for these sites, but also decreased substantially the intrinsic activity of the hormone.

Discussion

Our data show that monoiodoglucagon behaves identically with native glucagon in both its binding to specific glucagon binding sites in hepatic plasma membranes and on adenylate cyclase activity in these membranes. Since the preparation of monoiodoglucagon tested in this study was obtained from the same laboratory which reported the increased biological activity of monoiodoglucagon (Bromer et al., 1973), the difference between the two studies is not readily explainable. The tendency of glucagon to aggregate in aqueous solution (Gratzer and Beaven, 1969; Beaven et al., 1969) might be contributory to this discrepancy. We have found that the effectiveness of glucagon on adenylate cyclase activity decreases substantially after storage in solution, especially at concentrations above 10^{-5} *M*. Therefore, we have consistently used freshly prepared solutions of glucagon and the derivatives employed in this study.

It is essential to establish the purity of chemically modified hormones before any meaningful study can be carried out with the derivatives. We have taken great care to make certain that unreacted glucagon remaining after purification was minimal. Careful amino acid analyses indicated that contamination due to native glucagon was less than 0.5% for CNBr- and DH-glucagon. Therefore, the observed properties of these two derivatives cannot be attributed to

the unreacted glucagon. We have shown the extent of changes in the properties of glucagon produced by removal of two residues from the COOH-terminal region of glucagon by cyanogen bromide treatment. The contribution of Asn^{28} and Thr^{29} and possibly the terminal carboxy group to the affinity of the hormone is a major one. The participation of these hydrophilic residues (Nozaki and Tanford, 1971) indicates that hydrophobic interactions between glucagon and its receptor are not the only forces involved in the binding process. The parallel loss of both binding and biological activity strongly suggests that the role of the last two residues is strictly in the recognition process. They do not appear to be involved in the sequence of events leading to activation of adenylate cyclase.

It has been reported previously (Rodbell et al., 1971a) that DH-glucagon was devoid of biological activity with only a loss of about tenfold in affinity for the receptor. The implication of this finding and the potential use of this derivative as inhibitor of glucagon action are apparent (Birnbaumer and Pohl, 1973). Our study has confirmed the previous finding that the NH_2-terminal histidine residue in glucagon plays an important role in the expression of hormone action in addition to imparting some contribution to the binding of the hormone to the specific binding sites in hepatic plasma membranes. However, we found that DH-glucagon, prepared and purified as described here, is actually a weak agonist since, at 10^{-6} *M*, the derivative was able to induce about 70% of the maximal stimulation given by 10^{-8} *M* glucagon. Thus, the histidyl residue, though important in the binding and actions of the hormone, does not play an obligatory role in these processes. It is unlikely, therefore, that the histidyl residue of native glucagon participates in a catalytic function as observed for the histidine residue in various enzymes (Moore and Stein, 1973; Bender and Kezdy, 1965). It is possible that the histidyl residue is liganded to an important region of the receptor, or even the catalytic unit of adenylate cyclase, that is important in the expression of hormone action. In this regard, it has been shown that while dissociation of bound glucagon from its receptor is facilitated by the actions of guanine nucleotides on the adenylate cyclase system (Rodbell et al., 1971, 1974), binding of DH-glucagon to the receptor was relatively unaffected (Rubalcava and Rodbell, 1973). Since glucagon and guanine nucleotides activate adenylate cyclase in a concerted fashion (Rodbell et al., 1974), it is conceivable that the histidyl residue of glucagon is involved in this concerted process.

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