Roles of Aspartic Acid 15 and 21 in Glucagon Action: Receptor Anchor and Surrogates for Aspartic Acid 9[†]

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Received September 23, 1993; Revised Manuscript Received April 11, 1994[®]

ABSTRACT: The discovery of aspartic acid at position 9 in glucagon to be a critical residue for transduction has spurred renewed efforts to identify other strategic residues in the peptide sequence that dictate either receptor binding or biological activity. It also became apparent from further studies that Asp^9 operates in conjunction with His^1 in the activation mechanism that follows binding to the glucagon receptor. Indeed, it was later demonstrated that the protonatable histidine imidazole is important for transduction. It is likely that the interaction of a positively charged histidine 1 with a negatively charged aspartic acid 9 might be part of the triggering step at the molecular level. Two other aspartic acid residues in glucagon are capable of assuming a similar role, namely that of contributing to an electrostatic attraction with histidine via a negative carboxylate. These studies were conducted to investigate the role of aspartic acid 15 and 21 in glucagon action. Evidence reported here, gathered from 31 replacement analogs, supports the idea that in the absence of the requisite carboxyl group at position 9, histidine utilizes Asp^{21} or Asp^{15} as a compensatory site. Asp^{15} was also found to be indispensable for binding and may serve to tether the hormone to the receptor protein at the binding site. It is also demonstrated that these new findings promote the design of better glucagon antagonists.

Glucagon is a 29-residue peptide hormone that is secreted by the α cells of pancreatic islets. Its primary physiological role is to induce the breakdown of glycogen stored in liver and in adipose tissue whenever an organism is in need of glucose. The glucagon response is mediated by the second messenger cyclic AMP produced by activation of adenylate cyclase after the hormone binds to specific receptors in the cell membrane. The elevation of cyclic AMP above basal levels triggers an array of enzymatic reactions that result in glycogenolysis and a consequent rise in glucose output.

Glucagon is a well-studied peptide hormone, yet its mechanism of action at the molecular level and its contributing role to the metabolic abnormalities that accompany diabetes mellitus have not been delineated. Structure–function analysis has provided important information about the chemical and conformational properties required for receptor recognition and the subsequent expression of glucagon activity. The ability to segregate these two events has aided us in the design of glucagon antagonists that might be used in elucidating the molecular mechanism of hormone action and the role of glucagon in diabetes.

The identification of aspartic acid at position 9 as an essential determinant of glucagon activity, but not binding, resulted in a series of glucagon inhibitors (Unson et al., 1991a,b). Indeed, position 9 replacement analogs suffered markedly reduced potencies in adenylate cyclase activity, yet all bound with high affinities to the glucagon receptor. Removal of histidine 1 from these derivatives actually improved binding, while the analogs completely lost the ability to transduce the signal (Unson et al., 1991b). Earlier work had already indicated the prominant role of histidine 1 in the hormone response and showed the derivative des-His^1 glucagon to be a partial agonist (Lin et al., 1975; Bregman & Hruby, 1979). Further studies on position 1 showed evidence that the imidazole group of histidine 1 contributes a protonatable nitrogen that is important for activity (Unson et al., 1993). Results from position 1 and position 9 replacement analogs indicated that both histidine and aspartic acid must be present at the designated positions to achieve the maximum agonist response. These observations led us to conjecture that aspartic acid 9 and histidine 1 were somehow coupled electrostatically and that this attraction may be one molecular event in the mechanism of transduction.

In the absence of an aspartic acid carboxyl group at position 9, His^1-containing analogs remained weakly active, suggesting that despite the strategic importance of aspartic acid at position 9, histidine might be able to compensate for the missing negative charge by interacting with another carboxylic acid in the sequence. Interestingly, two other aspartic acid residues spaced six residues apart at positions 15 and 21 might be used as alternate sites.

A series of position 15 and 21 replacement analogs were synthesized to investigate which carboxylic side chain can behave as a surrogate for aspartic acid 9. We report here that both aspartic acid residues are responsible for partial agonism in the absence of the requisite position 9 aspartic acid. In addition, the carboxylic acid side chain of aspartic acid 15 but not aspartic acid 21 donates an essential feature for receptor recognition. These findings are significant for antagonist design, since a series of analogs that makes use of position 9 and 21 replacements are all antagonists with high binding affinities.

MATERIALS AND METHODS

Synthesis and Purification. A series of position 15 and 21 replacement analogs, some also as the des-His^1 derivatives, were synthesized by the solid-phase method (Merrifield, 1963; Barany & Merrifield, 1979) with 4-methylbenzhydrylamine resin (0.76 mmol/g) on an Applied Biosystems 430A peptide synthesizer. The peptide resins were treated with 50%

[†] This work was supported by U.S. Public Health Service Grant DK 24039.

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[®] Abstract published in *Advance ACS Abstracts,* May 15, 1994.

0006-2960/94/0433-6884$04.50/0

piperidine in dimethylformamide to remove the formyl protecting group on tryptophan prior to cleavage with HF. The crude peptides were extracted with 10% glacial acetic acid and purified as described previously (Unson et al., 1987). Purity of the peptide derivatives was verified by analytical HPLC, amino acid analysis, and mass spectral analysis which determined the $(M + H)^+$ peak to be within ±0.3 Da of theory.

Receptor Binding and Adenylate Cyclase Activity. Rat liver membranes were prepared from male Sprague-Dawley rats (Charles River Laboratories) by the Neville procedure as described by Pohl (1976), resuspended in 1 mM $NaHCO_3$, and stored as aliquots under liquid nitrogen until use. Protein was determined by a modified Lowry method (Markwell et al., 1978). The receptor binding assay was performed according to the procedure of Wright and Rodbell at equilibrium conditions (Rodbell et al., 1971; Wright & Rodbell, 1979), in 1% bovine serum albumin, 1 mM dithiothreitol, and 25 mM Tris-HCl buffer, pH 7.4. The amount of radioiodinated glucagon displaced from receptor sites by increasing concentrations of antagonist was measured. Binding affinity was expressed as the ratio of the concentration of natural glucagon to that of the antagonist required to displace 50% of receptor-bound labeled glucagon. Adenylate cyclase activity was measured by a protocol described by Salomon et al. (1974) in an assay medium containing 1% bovine serum albumin, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.025 mM GTP, 5 mM ATP, 0.9 mM theophylline, 17.2 mM creatine phosphate, and (1 mg/mL) creatine phosphokinase. The cAMP released was determined with a commercial kit from Amersham in which unlabeled cAMP was allowed to compete with [8-^{3}H]cAMP for a high-affinity cAMP-binding protein. Data for stimulation of adenylate cyclase are expressed as picomoles of cAMP produced per milligram of protein per minute and plotted against the logarithm of peptide concentration. Maximum activity is the ratio (×100) of the maximum response of analog to that of natural glucagon. Relative activity is a measure of the potency of the analog in the adenylate cyclase assay and is expressed as the ratio (×100), of the concentration of glucagon to that of the analog required to give half the maximum response of analog. This method of comparison was arbitrarily selected because the shapes of the activity response curves of natural glucagon and the analogs are not always parallel, especially in the case of partial agonists. An analog can retain partial agonist activity but relative to glucagon have very low potency.

Inhibition of adenylate cyclase was determined using the same procedure as above, except that increasing concentrations of antagonist were allowed to compete with a constant concentration of natural glucagon. The ratio of the concentration of antagonist to agonist when the response is reduced to 50% of the response to glucagon in the absence of antagonist is the inhibition index, $(I/A)_{50}$. The pA_2 value, calculated also from the dose–response curve (Arunlakshana & Schild, 1959), is the negative logarithm of the concentration of antagonist that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

RESULTS AND DISCUSSION

In the case of the hormone glucagon, structure–function analysis utilizing solid-phase peptide synthesis (Barany & Merrifield, 1979) has provided a method to sort out structural properties of the hormone that are important for binding from those that are necessary for activation. In spite of the onerous notion that the entire molecule is required for maximum potency in the agonist response, particular regions of the peptide that might influence the hormone's biological response more than receptor recognition have been defined in earlier work (Epand et al., 1981; Carrey & Epand, 1982; Flanders et al., 1984; Krstenansky et al., 1988). On the basis of these observations, superagonists (Krstenansky et al., 1986) and antagonists (Bregman & Hruby, 1979; Bregman et al., 1980; Gysin et al., 1987) of glucagon have been prepared. Moreover, the subsequent discovery that aspartic acid (Unson et al., 1991a,b) at position 9 primarily dictates the transduction function and the confirmation of the role of position 1 histidine (Unson et al., 1993) provided renewed confidence in the search for improved glucagon antagonists and greater insight into the mechanism of glucagon action.

Table 1: Position 15 Replacement Analogs

		adenylate cyclase activity			
analog of glucagon amide	binding affinity (%)	max activity[b] (%)	rel potency (%)	$(I/A)_{50}$	pA_2
glucagon amide	100	100	15		
(I) Leu^{15}	5.9	34	0.32	1071	5.6
(II) Nle^{15}	9.3	42.5	3.16	1412	5.0
(III) Gly^{15}	6.5	69	0.126		
(IV) Pro^{15}	1.3	28.5	0.56		
(V) Asn^{15}	7.1	81.3	1.78		
(VI) Ala^{15}	11.5	90	4		
(VII) Glu^{15}	79.4[a]	80	31.6		
(VIII) Lys^{15}	0.38	36.3	0.89		
(IX) D-Asp^{15}	16.2	100	100		
(X) des-His^{1}Leu^{15}	0.18	9.7	0.2		
(XI) des-His^{1}Nle^{15}	0.47	44	0.08		
(XII) des-His^{1}Gly^{15}	0.089	46.5	0.078		
(XIII) des-His^{1}Asn^{15}	0.24	30.6	0.056		
(XIV) des-His^{1}Ala^{15}	0.42	28.6	0.00012		
(XV) des-His^{1}Glu^{15}	17	30.7	0.158	132	6.2
(XVI) des-His^{1}Lys^{15}	0.31	36.3	0.4		
(XVII) Nle^{9}Nle^{15}	0.35	66	0.25		
(XVIII) Ala^{15}Leu^{21}	3.4	71	1.6		

[a] Glu^{9}glucagon amide had a binding affinity of 14% and a relative potency of 0.05% in the adenylate cyclase assay (Unson et al., 1991b).

[b] These maximum activities are obtained only at very high concentrations of analog.

At physiological conditions a protonated His^1-imidazole would be attracted to the first negatively charged residue in the sequence, aspartic acid 9. However, the peptide molecule is relatively small and flexible so that histidine should be able to interact with the other aspartic acid residues at positions 15 and 21, in the absence of aspartic acid 9. Indeed, we have observed that His^1-containing position 9 replacement analogs always retained partial agonist activity, which is predictably lost when histidine is removed. This also explains why His^1-containing position 9 analogs behaved only as weak inhibitors. We argued that histidine 1 must interact primarily with aspartic acid 9 but could use Asp^{15} or Asp^{21} as compensatory sites. The results of this investigation delineate the roles of both aspartic acid 15 and 21 in glucagon action and have important consequences for antagonist design.

Table 1 shows evidence that substitution of aspartic acid 15 had adverse effects on binding to the glucagon receptor. Replacement of the negative carboxyl group with a representative neutral moiety as in analogs **I**–**VI** effected a loss of about 90% binding. Reversal of charge in [Lys^{15}]glucagon amide (**VIII**) led to almost complete loss of receptor recognition. Transposing the carboxyl group to the opposite D-configuration in the analog [D-Asp^{15}]glucagon amide (**IX**) led to 84% loss of binding, but the analog elicited the maximum adenylate cyclase response. Only the similarly charged [Glu^{15}]-glucagon amide (**VII**) was tolerated by the receptor, being recognized with an 80% affinity relative to glucagon. Deletion

Table 2: Position 21 Replacement Analogs

analog of glucagon amide	binding affinity (%)	adenylate cyclase activity: max activity (%)	rel potency (%)	$(I/A)_{50}$	pA_2
glucagon amide[a]	100	100	15		
(**I**) Leu^{21}	263	95.2	100		
(**II**) Gln^{21}	29.5	100	100		
(**III**) Glu^{21}	89.1	100	100		
(**IV**) des-His^{1}Leu^{21}	19	11.6	0.52	20	8.1
(**V**) des-His^{1}Nle^{21}	21	14.3	0.044	81.6	7.2
(**VI**) des-His^{1}Glu^{21}	62	28.5	5.0	302	5.8
(**VII**) Glu^{9}Nle^{21}	13.2	15	0.45	78	7.6
(**VIII**) Nle^{9}Leu^{21}	10.5	27	1.58	18.2	8.3
(**IX**) Leu^{9}Leu^{21}	41.7	24.2	1.78	43.6	7.9
(**X**) des-His^{1}Glu^{9}Nle^{21}	7.1	0	<0.0004	91.4	6.7
(**XI**) des-His^{1}Nle^{9}Leu^{21}	34.7	0	<0.0015	2.1	8.7
(**XII**) des-His^{1}Glu^{9}Glu^{21}	224	0	<0.005	4.25	8.2
(**XIII**) des-His^{1}Nle^{9}Glu^{21}	48	0	0.0166	6.92	8.1

[a] Des-His^{1}glucagon amide had a binding affinity of 63% and a relative potency of 0.16% in the adenylate cyclase assay, and Nle^{9}glucagon amide had a binding affinity of 32% and a relative potency of 0.17% (Unson et al., 1991b).

of position 1 histidine reduced binding potencies even further. Interestingly, despite unfavorable effects on binding, the analogs were partial agonists, although with decreased potencies. For example, it required 300 times the amount of [Leu^{15}] (**I**) to achieve a maximum activity of 34% and 800 times the concentration of [Gly^{15}] (**III**) to obtain a maximum activity of 69% that of natural glucagon. The exceptions were [Glu^{15}]glucagon amide (**VII**) with a maximum activity of 80% and a relative activity of 31.6% and [D-Asp^{15}]glucagon amide (**IX**), a full agonist, that was as potent as glucagon. Thus, the decreases in activity roughly followed the decreases in receptor binding, and it was thus not surprising that none of the position 15 replacement analogs were good glucagon inhibitors.

In the case of position 21, the results listed in Table 2 were strikingly different. Most of the replacement analogs retained strong binding affinities. The analogs [Leu^{21}]-, [Gln^{21}]-, and [Glu^{21}]glucagon amides (analogs **I–III**) bound 263%, 29.5%, and 89.1%, respectively, and were as potent as the natural hormone amide in the adenylate cyclase assay with a full agonist response. The corresponding des-His^1 position 21 derivatives analogs **IV–VI** still bound very well but instead had strongly adverse effects on activity. For example, des-His^1[Nle^{21}]glucagon amide (**V**) was a partial agonist with a maximum activity of 14.3% but required 2300 times the amount to achieve that response. The pattern was slightly reminiscent of the way position 9 responded to modification. Thus, as at position 9, position 21 aspartic acid contributes to signal transduction but not to receptor binding. All of the analogs containing replacements at both Asp^9 and Asp^{21} exhibited weak activity, and they were completely inactive when His^1 was also absent. Subsequently, analogs **X–XIII** in Table 2 that displayed high binding and no adenylate cyclase activity were pure antagonists, and analogs **IV–IX** which had preserved good binding affinities and were partial agonists with low relative potencies also behaved as inhibitors (Hruby et al., 1986). The presence of Asp^9 in the position 21 replacement analogs sustained transduction as expected, which explains why these derivatives were as potent as the natural hormone. Indeed, this was consistent with the finding that a glutamic acid replacement at position 21 contributed to superagonism in Lys^{17}Lys^{18}Glu^{21} (Krstenansky et al., 1986). Des-His^1[Glu^{21}]glucagon amide (**VI**) bound 62% but had a relative potency of 5% in the adenylate cyclase response. Unlike des-His^1[Glu^9]glucagon amide, des-His^1[Glu^{21}]glucagon amide, a partial agonist (28% maximum activity), was a poor antagonist. We can argue that the predominance of Asp^9 in the derivative des-His^1[Glu^{21}]glucagon amide would still allow it to be an agonist at high concentrations, which is also consistent with the properties of the present antagonist des-His^1 glucagon amide (Merrifield et al., 1987).

The data demonstrate a peculiar phenomenon that refines and expands our view of how the structure of glucagon relates to its biological activity. Unlike a more rigid globular protein, a medium-sized peptide like glucagon is flexible enough to fold into a spectrum of conformations that the receptor might allow into its binding pocket. Not all of these configurations will trigger the step that leads to transduction. However, the peptide molecule is small enough so that many of the residues could be within a distance sufficient for even weak interactions. These smaller forces of attraction that hold the polypeptide into the proper fold may actually contribute to the overall binding affinity and subsequent activation. A single modification would rarely result in a completely inactive hormone, which is one reason why structure–activity studies are difficult to interpret, and the observed properties of each analog are usually not a linear summation of the effects of the individual changes. Furthermore, although we presume better intuitive correspondence between binding affinities and inhibitor potency, we as well as others have observed that in some analogs it is not always the case (Zechel et al., 1991). These observations are not entirely unexpected since the nonlinear relationship between binding of the hormone and the stimulation of cyclic AMP production, with nearly full activation even before steady-state binding is achieved, has been demonstrated for intact hepatocytes (Sonne et al., 1978) and for liver plasma membranes (Birnbaumer et al., 1972; Birnbaumer & Pohl, 1973). In addition, glucagon receptor heterogeneity is well accepted, but whether there are distinct classes of binding entities linked to different effectors (Wakelam et al., 1986) or one form of a glucagon receptor existing in two (or more) different states (Horwitz et al., 1986; Bharucha & Tager, 1990) regulated by complex binding equilibria is still in question. Horwitz et al. (1986) have suggested that conversion of hormone/receptor complexes from a low-affinity state to a high-affinity state is required for the activation of adenylate cyclase. More recently, the mechanism of action of the antagonist des-His^1[Glu^9]glucagon amide (Unson et al., 1987) has been shown to involve binding to the low-affinity GTP/Mg^{+2}-independent receptor states that do not lead to cyclase activation (Post & Tager, 1993).

This analysis of the role of aspartic acid residues in glucagon provides a clearer understanding of some of the electronic interplay that is critical to glucagon action. It confirms that aspartic acid 9 is indeed the crucial residue for activity and is a point of uncoupling of receptor binding function from signal transduction. Transduction will still occur even in the absence of the aspartic acid carboxyl at position 21, while greater than 96% of the relative potency of cAMP production is lost, consistent with a accompanying decrease in receptor binding, without a carboxyl group at position 15. In the absence of the principal carboxyl group at position 9, histidine can therefore relay on an existing supporting interaction with aspartic acid 15 or 21. This conclusion was drawn from the observation that His^1-containing position 9 analogs still elicited a residual adenylate cyclase response. In addition, more compelling evidence demonstrated that analogs that combined replacements on both positions 9 and 21 (**VII–IX**; Table 2) maintained the capacity for cAMP production above basal (15–27% partial activity), although with reduced potency, because of the presence of His^1 and Asp^{15}. Similarly, in Table 1, the combined replacement of positions 9 and 15 in Nle^9-Nle^{15} analog (**XVII**) did not completely extinguish the cAMP response, despite a drastic loss in binding affinity. Substitution of both surrogate residues in Ala^{15}Leu^{21} analog (**XVIII**) produced a partial agonist (71% maximum activity) with reduced potency associated with decreased binding.

Because the observed cAMP activity at position 15 was closely dependent on the binding strength, its contribution to the overall biological response was not clear. Nonetheless, our results indicate that Asp^{15} has an important function in receptor recognition. The negatively charged carboxyl group is important for binding since only aspartic acid and glutamic acid were well tolerated by the receptor at this site. We can reason that although Asp^{15} sustains electrostatic interaction with His^1, its more important role is most likely to interact with a complementary site in the receptor protein. This intermolecular electrostatic force helps tether and position the hormone into the binding pocket.

The principal roles inferred for the two aspartic acid residues other than Asp^9 in glucagon, that of a counterion docking point with the receptor in the case of Asp^{15} and that of a surrogate for Asp^9 during transduction in the case of Asp^{21}, augment our perception of the electronic and conformational features that govern glucagon action. The physiological configuration one envisions for the hormone is remarkably consistent with the Korn and Ottensmeyer working model for glucagon (Korn & Ottensmeyer, 1983). In this structure, both Asp^9 and Asp^{21} are topologically juxtuposed so that His^1 is capable of an interaction with both residues. Asp^{15} is less favorably situated for the same intramolecular interaction with His^1. Instead, Asp^{15} is positioned at a turn where the peptide folds. One can assume that the carboxyl group must protrude out of the kink and is available to function as a hinge that assists in anchoring the ligand to its receptor.

Thus, we have deduced the roles of two other sites within the sequence of glucagon, that of Asp^{15} for receptor binding, and a secondary role in supporting biological activity, and that of Asp^{21} primarily as an compensatory residue for transduction. These new findings dispel the notion that the binding function of the sequence is delegated to the carboxyl end of the molecule and transduction toward the amino terminus. The receptor requires the entire peptide for recognition and for full expression of the biological response. Thus, it becomes highly unlikely that truncated forms of glucagon will come close to mimicking the natural hormone. These novel observations not only provide greater insight into glucagon receptor interaction but also advance our capacity to design more potent glucagon antagonists. Combinations of position 9 and 21 replacement analogs as well as their des-His^1 derivatives are all pure glucagon antagonists, with pA_2 values ranging from 6.7 to 8.7 and inhibitor indices as low as 2.1 (Table 2). Our results argue for continued structure–function studies that may, in the absence of X-ray structure of a glucagon/receptor complex, gradually elucidate the molecular events that occur in the glucagon signaling system.

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