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# Identification of an essential serine residue in glucagon: Implication for an active site triad

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**ABSTRACT** **Several glucagon analogs containing substitutions for serine have been synthesized to assess the role of the four serine residues in the hormone. The strategic importance of His[1] has been confirmed, and we have previously identified an aspartic acid critical for activity at position 9. While these findings have led to a series of pure glucagon antagonists, the details of specific glucagon–receptor interactions that switch on the ensuing signaling events are still not readily apparent. The requirement for serine was tested by the chemical synthesis of a series of analogs containing substitutions for the hydrophilic hydroxyl group in each of the highly conserved serine residues at positions 2, 8, 11, and 16 of glucagon. The resulting analogs were analyzed in rat hepatocyte membranes for their receptor-binding affinities as well as their abilities to stimulate adenylate cyclase. Positions 2 and 8 were the most sensitive to modification, where both binding and activity were adversely affected. This is consistent with the notion that although the sequence responsible for transduction lies in the amino-terminal half of glucagon, some residues at that end also contribute to binding affinity. Modifications at position 11 generated high-binding-affinity derivatives that were full or moderate agonists. In contrast, position 16 replacement analogs maintained significant receptor binding affinities while the agonist properties were almost completely lost, thus separating binding and transduction functions. Therefore, Ser[16] is a third critical residue that determines glucagon activity. It is postulated, but not proven, that a serine residue, together with His[1] and Asp[9], may participate in the putative active center of glucagon, which, upon initial recognition and binding to receptor, leads to transduction of the biological signal. A dependence of the glucagon action on a three-residue cooperative mechanism might be analogous to the charge-relay scheme of serine proteases. It is suggested that, after binding to its receptor, glucagon becomes activated and functions like a coenzyme in catalyzing the specific hydrolysis of a peptide bond in the receptor, generating new amino and carboxyl end groups, and that one of these exposed chains may contact the GTP-binding protein and activate it for further interaction with adenylate cyclase. This idea was supported by inhibition experiments with 4-amidinophenylmethanesulfonyl fluoride (APMSF), a specific and irreversible inhibitor of serine proteases, which at a concentration of 5 mM completely suppressed cAMP formation by glucagon in liver membranes. cAMP formation was not affected if either glucagon or membranes were separately pretreated with APMSF and then assayed.**

Glucagon is a peptide hormone of the pancreas (containing 29 amino acid residues), which, together with insulin, is an important regulator of glucose metabolism. When an animal is in need of glucose, glucagon is secreted by the A cells of the pancreas and binds to target cell-surface receptors in the liver. This triggers a series of events that ultimately results in glycogenolysis and gluconeogenesis and a consequent rise in glucose levels in the blood.

The driving force for continued efforts to study glucagon lies primarily in the still unresolved problem of diabetes mellitus. Data accumulated in recent years leave little doubt that in insulin-deficient diabetes, elevated plasma glucagon levels exacerbate the metabolic abnormalities of the disease (1). These considerations imply that antagonists of the hormone that are able to inhibit the actions of this endogenous glucagon could be a potentially useful adjunct to insulin therapy in the management of diabetic hyperglycemia.

The glucagon-sensitive adenylate cyclase system consists of functionally and structurally distinct units: a receptor protein, linked by the stimulatory GTP-binding protein (Gs) to the catalytic subunit of adenylate cyclase, which converts ATP to cAMP (2). It is well accepted that hormone occupation of the receptor activates the Gs protein and a cascade of enzymatic events that constitutes transduction of the biological signal. The glucagon receptor is a 63-kDa transmembrane glycoprotein, which can be cleaved by proteolytic enzymes to give 33-kDa and 24-kDa products that retain the ability to bind glucagon (3). Receptor-linked proteolysis of membrane-bound glucagon, yielding a membrane-associated hormone fragment, was also reported (4). However, the mechanism by which the glucagon signal is transmitted from the receptor to the effector system in the hepatocyte membrane is not known. It is known that an activator region of the $\beta_2$-adrenergic receptor and a synthetic peptide corresponding to this region can activate the GTP-binding protein (G protein) coupled to this receptor (5). It was also shown recently that thrombin activates its own receptor by hydrolysis at the LDPR/S site and that a short synthetic peptide corresponding to this region could activate the G protein (6). In addition, it was previously demonstrated that the insulin receptor, when nicked by proteolytic enzymes, remains fully active (7). The roles of G proteins, GTP, $Mg^{2+}$, and other factors in the transduction of hormonal signal have been reviewed (8).

Recently, we identified the aspartic acid residue at position 9 of glucagon as a critical residue for the transduction effect but not for binding to receptor (9, 10). An uncoupling of the binding interaction from adenylate cyclase activation was demonstrated by the observation that amino acid replacements at position 9 resulted in peptides with binding affinities ranging from 10% to 100% relative to natural glucagon and yet had no measurable effect on production of cAMP above basal in rat liver membranes. Only aspartic acid at position 9 was active. These Asp[9] substitution analogs proved to be a series of potent antagonists *in vitro* (10), and some were shown to be antagonists of glucagon *in vivo* (11).

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Abbreviations: G protein, GTP-binding protein; Gs, stimulatory GTP-binding protein; APMSF, 4-amidinophenylmethanesulfonyl fluoride; DIFP, diisopropylfluorophosphate; $(I/A)_{50}$, inhibition index; $pA_2$, negative logarithm of the concentration of antagonist that reduces the response toward 1 unit of agonist to the response obtained from 0.5 unit of agonist.

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Just as strategic, however, is the role that the $His^1$ residue plays in glucagon function (12). The role of a requisite imidazole group at position 1 was more thoroughly investigated, and we determined that a series of position-1 substitutions also brought about attenuation of the cAMP response more than it did receptor binding (13). We argued that perhaps $His^1$ and $Asp^9$ in glucagon were interacting electrostatically to stabilize a conformation that leads to activation of Gs and, subsequently, adenylate cyclase.

In our continued efforts to identify structural determinants in the hormone that are responsible for activity, it was not unreasonable to speculate that a serine residue might cooperate with $His^1$ and $Asp^9$ to produce an active center. Such a putative His, Asp, Ser active site is reminiscent of the serine protease triad. Glucagon contains four serine residues, at positions 2, 8, 11, and 16, which are conserved among the members of the family. The requirement for serine was tested by a series of substitutions for the hydrophilic hydroxyl group in each of the four positions. The resulting analogs were analyzed for their receptor binding affinities as well as their abilities to stimulate adenylate cyclase in hepatocyte membrane preparations.

## MATERIALS AND METHODS

**Synthesis and Purification.** Serine replacement analogs at positions 2, 8, 11, and 16 were synthesized by the solid-phase method (14, 15) on an Applied Biosystems 430A peptide synthesizer as described (16). After *t*-butoxycarbonyl deprotection, the peptide resins were treated with 50% (vol/vol) piperidine in *N,N*-dimethylformamide to remove the formyl group on tryptophan prior to high HF cleavage from the resin support. The integrity of the peptide derivatives was verified by analytical HPLC, amino acid analysis, and mass spectral analysis, which determined the $(M + H)^+$ peak to be $\pm 0.3$ Da of theory.

**Receptor Binding and Adenylate Cyclase Activity.** Liver membranes were prepared from male Sprague–Dawley rats (Charles River Breeding Laboratories) by the Neville procedure as described by Pohl (17), resuspended in $NaHCO_3$, and stored as aliquots under liquid nitrogen until use. Protein was determined by a modified Lowry method (18). The receptor binding assay was performed according to the procedure of Wright and Rodbell (19). 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 times 100. Activation of adenylate cyclase in rat liver membranes was measured by a procedure described by Salomon *et al.* (20). 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. Relative potency is reported as 100 times the ratio of the concentration of glucagon to that of the analog to give 50% of maximum cAMP response. For low-activity analogs that never reach the 50% level, we have compared the concentrations of glucagon and the analog that give 50% of the maximum response of the analog. Inhibition of adenylate cyclase was determined by using the same procedure, except that increasing concentrations of antagonist were allowed to compete with a constant concentration of natural glucagon. A level of glucagon was chosen that gives ≈80% of maximum response. The ratio of the concentration of antagonist to that of the 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 (21) is the negative logarithm of the concentration of antagonist that reduces the response toward 1 unit of agonist to the response obtained from 0.5 unit of agonist.

**Inhibition with 4-Amidinophenylmethanesulfonyl Fluoride (APMSF) and Diisopropylfluorophosphate (DIFP).** Production of cAMP in hepatocyte membranes by increasing concentrations of natural glucagon was measured in the presence of 1–10 mM APMSF or 1–20 mM DIFP. The response was compared to a standard protocol in the absence of protease inhibitor.

## RESULTS

Thirty-eight peptide analogs that contained replacements for serine at position 2, 8, 11, or 16 were synthesized and tested for receptor binding and adenylate cyclase activity. This strategy sought to discover a critical serine residue predicted to be necessary for transduction of the glucagon signal. The data listed in Table 1 demonstrate that each of the serine residues plays a varying yet distinct functional role in glucagon. Positions 2 and 8 at the amino-terminal end of the peptide were very sensitive to modification. Analogs 2–23 suffered a marked loss in both receptor binding and adenylate cyclase activity. Surprisingly, only the substitution of an isosteric alanine or the hydroxyl-bearing threonine at position 2 produced analogs that still retained fairly good binding affinities of 45% in the case of $Ala^2$ (no. 2) and 100% in the case of $Thr^2$ (no. 3) and were capable of eliciting a high cAMP response. The D-serine analogs at positions 2 and 8 also adversely affected binding (nos. 4 and 16). At these sites it is evident that molecular dimension and configuration are determinants for binding, which in turn is closely linked with activity. Replacement at position 2 with residues other than alanine or the hydroxyl-bearing threonine (analogs 4–6) and at position 8 with amino acids other than alanine (analogs 13–16) provided poor binders with a correspondingly weakened relative potency.

In contrast, Table 1 shows that substitutions for serine 11 had little adverse effect on binding. Replacement of the hydroxyl group at position 11 with an isosteric methyl group or a β-branched hydroxyl yielded the analogs [$Ala^{11}$]glucagon amide (no. 24), which bound 4 times better than natural glucagon, and [$Thr^{11}$]glucagon amide (no. 25), which bound 2.5 times better. Even the inversion of the hydroxyl-containing side chain by substituting a D-serine at position 11 produced an analog (no. 27) that recognized receptor almost as effectively as natural glucagon. $Asn^{11}$ (no. 26) and $Phe^{11}$ (no. 28) were fairly good binding analogs, in which a slight loss in affinity may have been due to unfavorable steric effects. Position 11 replacement analogs 24–28 were potent agonists with high maximum activities relative to natural glucagon.

Analogs bearing serine modifications at position 16 (nos. 33–36, Table 1) also retained receptor binding affinities that were as strong as the native hormone. An exception was the β-branched replacement in [$Thr^{16}$]glucagon amide (no. 36), which demonstrated that a bulky side chain was unfavorable at this site. However, while changes at position 16 did not generally alter high-affinity analog binding, adenylate cyclase activation was seriously impaired. Position-16 analogs were weak partial agonists. The absence of the hydroxyl group in $Ala^{16}$ allowed 100% binding but brought about a reduction of relative potency to only 0.89%. Replacement with D-$Ser^{16}$ gave similar results. In the case of $Thr^{16}$, which contained additional steric bulk, a reduction in binding to 8.1% and relative activity to 0.43% was observed. The corresponding des-$His^1$ derivatives (nos. 38–40), while displaying reduced binding, suffered even further reduction in potencies. These results are an indication of a separation of binding and transducing functions at position 16 and provide evidence for an important role for $Ser^{16}$ in transduction.

Table 1. Position 2, 8, 11, and 16 replacement analogs

| Analog of glucagon amide | Binding affinity, % | Adenylate cyclase activation | | |
|---|---|---|---|---|
| | | Relative potency, % | $(I/A)_{50}$ | $pA_2$ |
| 1. Glucagon amide | 100 | 15 | — | — |
| 2. [$Ala^2$] | 45 | 63 | — | — |
| 3. [$Thr^2$] | 100 | 39 | — | — |
| 4. [D-$Ser^2$] | 12 | 36 | — | — |
| 5. [$Tyr^2$] | 4 | 0.31 | 135 | 6.2 |
| 6. [$Phe^2$] | 0.7 | 0.2 | — | — |
| 7. des-$His^1$-[$Ala^2$] | 1.4 | 0.33 | — | — |
| 8. des-$His^1$-[$Thr^2$] | 8.7 | 0.5 | 125 | 6.5 |
| 9. des-$His^1$-[D-$Ser^2$] | 1.7 | 0.26 | 107 | 6.6 |
| 10. des-$His^1$-[$Tyr^2$] | 3.7 | 1.8 | 44.5 | 6.8 |
| 11. des-$His^1$-[$Phe^2$] | 3.2 | 0.17 | 135 | 6.4 |
| 12. [$Ala^8$] | 13 | 4 | — | — |
| 13. [$Gly^8$] | 3.2 | 4 | — | — |
| 14. [$Thr^8$] | 1.0 | 3.4 | 323 | 4.6 |
| 15. [$Asn^8$] | 8.0 | 4.7 | — | — |
| 16. [D-$Ser^8$] | 1.4 | 1.1 | — | — |
| 17. des-$Ser^8$ | 4.0 | <0.013 | 54 | 6.8 |
| 18. des-$His^1$-[$Ala^8$] | 3.2 | 0.71 | 170 | 5.8 |
| 19. des-$His^1$-[$Gly^8$] | 3.2 | <0.0022 | 15 | 7.0 |
| 20. des-$His^1$-[$Thr^8$] | 0.1 | 0.45 | — | — |
| 21. des-$His^1$-[$Asn^8$] | 0.14 | 0.074 | 142 | 6.2 |
| 22. des-$His^1$-[D-$Ser^8$] | 0.9 | 0.41 | — | — |
| 23. des-$His^1$, $Ser^8$ | 0.3 | <0.007 | 41 | 6.4 |
| 24. [$Ala^{11}$] | 400 | 41 | — | — |
| 25. [$Thr^{11}$] | 250 | 100 | — | — |
| 26. [$Asn^{11}$] | 25 | 22 | — | — |
| 27. [D-$Ser^{11}$] | 63 | 29 | — | — |
| 28. [$Phe^{11}$] | 12 | 50 | — | — |
| 29. des-$His^1$-[$Ala^{11}$] | 10 | <0.006 | 27 | 6.9 |
| 30. des-$His^1$-[$Thr^{11}$] | 8 | 1.8 | 2042 | 5.5 |
| 31. des-$His^1$-[$Asn^{11}$] | 1.3 | 0.39 | 213 | 5.4 |
| 32. des-$His^1$-[D-$Ser^{11}$] | 1 | 0.12 | 159 | 6.0 |
| 33. [D-$Ser^{16}$] | 100 | 6.9 | — | — |
| 34. [$Ala^{16}$] | 100 | 0.89 | — | — |
| 35. [$Gly^{16}$] | 100 | 5.4 | — | — |
| 36. [$Thr^{16}$] | 8.1 | 0.43 | 54.7 | 6.8 |
| 37. des-$His^1$ (Native) | 63 | 0.16 | | |
| 38. des-$His^1$-[$Ala^{16}$] | 11 | 0.048 | 43.4 | 6.8 |
| 39. des-$His^1$-[$Gly^{16}$] | 4.8 | <0.0012 | 152 | 6.2 |
| 40. des-$His^1$-[$Thr^{16}$] | 1.6 | <0.0044 | 135 | 6.5 |

Identification of an essential serine residue together with the established roles of $His^1$ and $Asp^9$ brought to mind the His, Asp, Ser catalytic triad of serine proteases. To examine whether or not an activated serine was present in the glucagon–receptor complex, glucagon in the presence of the hepatocyte membrane was treated with 20 mM DIFP at 25°C for 30 min or with 5 mM APMSF at 30°C for 30 min. The former reduced the adenylate cyclase response to 22%, and the latter effectively inhibited glucagon-stimulated adenylate cyclase activity to less than 0.1% (Fig. 1).

As controls to determine which component of the system was sensitive to the serine protease inhibitors, natural glucagon was preincubated with either 10 mM APMSF or 20 mM DIFP for 30 min at 30°C, gel-filtered to remove the inhibitor, mixed with liver membranes, and assayed for adenylate cyclase activity. An aliquot of freshly isolated rat hepatocyte membranes was treated similarly for 10 min, washed to eliminate inhibitor, mixed with glucagon, and assayed for the viability of its glucagon adenylate cyclase system compared to membranes that had not been incubated with serine protease inhibitors (Fig. 2). It was found that treatment of glucagon alone with APMSF prior to the cyclase assay did not affect its ability to stimulate adenylate cyclase in hepatocyte membranes. Likewise, a membrane preparation, containing the glucagon receptor, the G protein, and adenylate cyclase but not glucagon, did not lose its ability to generate cAMP after exposure to high concentrations of APMSF or DIFP at two different temperatures and times when assayed in the presence of added glucagon, although at the longer time a small decrease was observed. In contrast, treatment of a mixture of glucagon plus membranes with APMSF reduced the production of cAMP to baseline levels (Fig. 2). Thus, attenuation of adenylate cyclase activity by serine protease

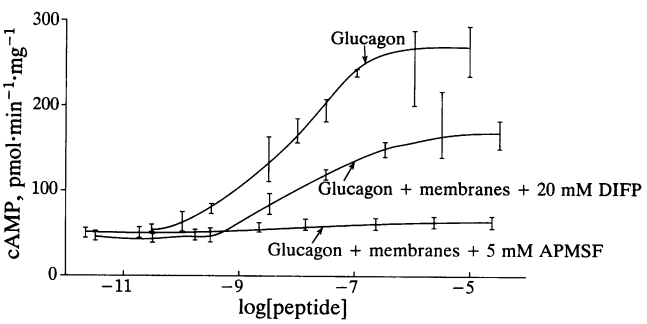


FIG. 1. Inhibition of cAMP formation in glucagon-stimulated rat liver membranes by serine protease inhibitors.

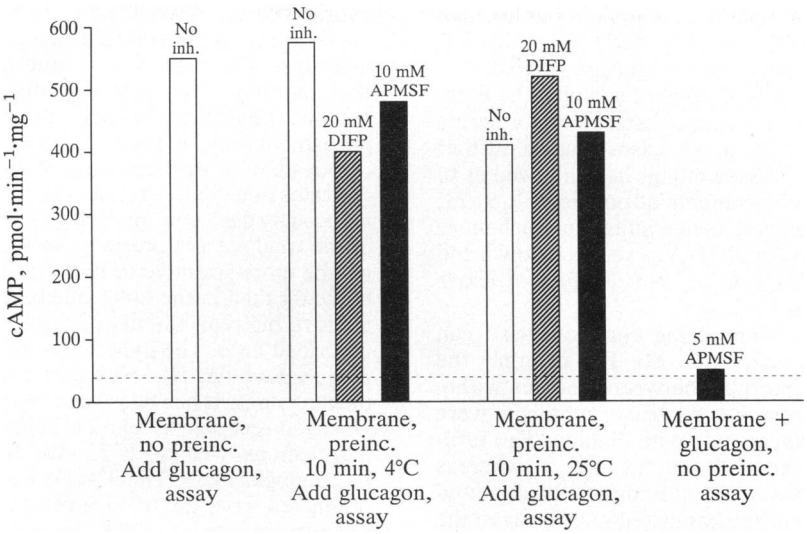


FIG. 2. Stability of membranes toward serine protease inhibitors. The dotted line is basal cAMP in the absence of glucagon. preinc., preincubation. inh., inhibitor

inhibitors was observed only during the simultaneous presence of both the hormone and its receptor system.

Glucagon plus membranes was incubated for 1 hr at 25°C with [$^3$H]DIFP and run on a Sephadex G-50 column (1 × 48.3 cm) in 0.1 M HOAc. The fractions were measured at 220 nm and aliquots were counted for $^3$H. Tritium label was located at the position of glucagon and at the position of excess DIFP, but none was found in the excluded volume where the protein components of the assay system were eluted. Since the excess DIFP fractions overlapped with the peptide fraction, all the tubes containing label were combined, lyophilized, and applied to a G-15 column (1 × 28.5 cm). The fractions were measured at 220 nm and counted for tritium. The DIFP fractions were well separated from glucagon fractions, with no measurable overlap. The 220-nm chromatogram showed two small peaks near the position of glucagon that were both tritium-labeled, suggesting that $^3$H-labeled peptide was produced in the experiment. Amino acid analysis of these fractions indicated peptide material.

## DISCUSSION

It is well-accepted that virtually the entire sequence of glucagon is necessary for the full expression of hormonal activity. However, in early studies (19), the fragment glucagon-(1–6) was shown to be partially capable of stimulating adenylate cyclase and led to the notion that the amino-terminal half of the molecule was responsible for transduction, whereas the carboxyl sequence conferred receptor specificity and binding affinity. Structure–activity work demonstrated that histidine at position 1 contributed significantly to glucagon activity, so that the analog des-His$^1$-glucagon, a weak partial agonist, was also the first known antagonist of this hormone (12).

Recently, the discovery that the Asp$^9$ residue was absolutely required for glucagon activity (9, 10) bolstered the concept of transduction being dictated by particular residues toward the amino terminus. Asp$^9$ proved to be a locus of uncoupling of the binding function from transduction. Moreover, a combination of the des-His$^1$ feature with a position-9 substitution led predictably to a series of analogs that were still recognized by the glucagon receptor but did not activate cyclase. Taken together, these results gave strong credence for the essential roles of both His$^1$ and Asp$^9$ in glucagon action and the proposal that an interaction between them and some putative site at the receptor protein leads to cyclase activation (10). All of these des-His$^1$ position-9 replacement analogs proved to be glucagon antagonists with moderate to good inhibition indices (10).

Other key amino acid residues that may be involved in glucagon activity had not been identified. Nevertheless, the idea that a histidine and an aspartic acid were interacting in a cooperative manner led us to conjecture that a topologically proximal serine might provide the missing component in the transmission of the biological message, reminiscent of the His, Asp, Ser triad of serine proteases. Our speculations were reinforced by the observation that APMSF and, to a lesser extent, DIFP effectively inhibited glucagon-stimulated adenylate cyclase in liver membranes (Fig. 1).

In glucagon, any one of the four highly conserved serine residues at positions 2, 8, 11, and 16 might associate with His$^1$ and Asp$^9$ in the generation of an active center. It is believed that an oxyanion, generated from the hydroxyl of a serine residue in the serine proteases through abstraction of the proton by a histidine in a charge relay network, carries out a nucleophilic attack on the substrate. A negative aspartate stabilizes the positively charged histidine in the transition state.

Our results provide evidence that serines 2, 8, and 11 can affect binding but are not involved in transduction, because the observed cAMP activity varied approximately as a function of binding strength. For example, Ala$^2$ retained full binding and high activity, Ala$^8$ gave weaker binding and lower activity, whereas Ala$^{11}$ elicited very high binding and retained good activity. Since the absence of the requisite hydroxyl group in these analogs did not affect glucagon activity more than binding affinity, we concluded that serines 2, 8, and 11 are not required for participation in a charge relay network similar to that of serine proteases. These studies make evident that the hydroxyl group of serine-8 contributes important structural determinants for glucagon binding and may provide additional stabilization by hydrogen bonding at the binding site. The serine residues at positions 2 and 11, however, do not appear to be essential for binding. These results also verify that the binding site of the hormone does not preclude a role for residues at the amino terminus and that there is no clear segregation of a transduction region from a receptor binding region.

Position-16 replacement analogs, on the other hand, presented a markedly different sensitivity to alteration. Very much like replacements for His$^1$ and Asp$^9$ (10, 13), most substitutions for Ser$^{16}$ (analogs 33–36; Table 1) strongly

attenuated adenylate cyclase activation while retaining high binding affinity. These results identify $Ser^{16}$ as a third important residue for transduction of the glucagon signal.

Consistent with position-9 replacement analogs, the combined removal of $His^1$ together with substitutions for serine at positions 2, 8, 11, and 16 in all cases caused further attenuation of the activity. These findings have allowed us to design analogs in which replacements at positions 1, 9, 11, and 16 have been combined and have resulted in much more potent glucagon antagonists, with $(I/A)_{50}$ values below 1 and $pA_2$ values up to 8.8 (C.G.U., C. R. Wu, K. Fitzpatrick & R.B.M., unpublished data).

Serine protease analogs without the complete triad can retain some proteolytic activity (22, 23). For example the catalytic importance and interplay between residues within the catalytic triad of the serine protease subtilisin were examined by multiple replacements with alanine. The turnover number was greatly reduced, but not to zero, whereas the binding of substrate was not. In addition, the background hydrolysis was about 25% of the catalyzed rate for even the least active derivatives. This is attributed to water, which can function directly as a nucleophile. It was concluded (23) that an intact catalytic triad is not an absolute requirement for peptide bond cleavage by trypsin analogs. Our finding that position-16 analogs retained some activity and strong binding may illustrate a similar phenomenon.

Although it is presumed that the residues responsible for transduction are largely located toward the amino terminus, the critical serine is situated at the center of the hormone in one of the predicted $\beta$-turns. Using the working model of glucagon in dilute solution proposed by Korn and Ottensmeyer (24), we find that $His^1$, $Asp^9$, and $Ser^{16}$ side chains, based on computer graphics, can be juxtaposed to create the hypothetical charge relay triad being considered. This proposed hydrogen transfer network and the formation of a reactive serine hydroxyl should be disrupted by serine protease inhibitors. Experiments described in Fig. 1 in which glucagon together with hepatocyte membranes were incubated in the presence of serine protease inhibitors showed marked inhibition of cAMP production with 20 mM DIFP and complete suppression with 5 mM APMSF. Separate preincubation of either natural glucagon or a hepatocyte membrane preparation with 10 mM APMSF or 20 mM DIFP did not markedly affect adenylate cyclase activity (Fig. 2).

Assuming that an important component of the membranes, the glucagon receptor, the G protein, or the cyclase itself, was susceptible to these inhibitors, the unusually high concentrations of enzyme inhibitor to which the membranes were exposed should have been sufficient to abolish cAMP production. These control experiments established that complete inhibition by serine protease inhibitors of the glucagon-induced cAMP response occurs only when both agonist and receptor system were present together. The data fit the idea that glucagon *in the presence of its receptor* assumes a conformation in which $Ser^{16}$ is activated sufficiently to function as the active nucleophile of a protease. The activated serine would also be susceptible to inactivation by DIFP or APMSF. The experiments with tritiated DIFP showed that the radioactivity of this inhibitor became associated with the glucagon fraction, not the receptor component, of the glucagon–receptor complex and support the idea that a serine, probably $Ser^{16}$, may be at the active site of the protease and was covalently labeled by the inhibitor.

We propose that the glucagon–receptor complex acquires enzyme activity upon formation and that its activation might initiate the transduction step in the glucagon-sensitive adenylate cyclase system. Formation of the glucagon receptor complex would be viewed as doing two things: it would align the Asp, His, Ser triad of the hormone for catalysis of amide bond hydrolysis; and it would serve to position a sensitive bond in the receptor for enzymatic cleavage. Thus, the enzyme would hydrolyze a specific bond in the receptor with liberation of new carboxyl and amino groups. This might modify the receptor conformation and activate it for reaction with G protein, or one of these exposed chains might be directly responsible for the activation of the G protein, which in turn activates adenylate cyclase and the cascade of events that is induced by glucagon. In analogs where all three of the residues of the triad are not present, the susceptible amide bond may still be more sensitive to hydrolysis by other nucleophiles or by water than in the unliganded receptor. This might explain the low, but real, activity of some of the replacement analogs described here. The hypothesis may also explain antagonism in the case of des-$His^1$-glucagon amide and position-9 replacement analogs. It has been reported in a structural study of the glucagon receptor by photoaffinity labeling (3) that a 64-kDa fragment as well as a 33-kDa fragment are labeled with [$^{125}$I]iodoglucagon. The 33-kDa fragment was shown to have originated from the 64-kDa receptor still bound to glucagon and was sensitive to GTP. An agonist bound to the receptor may lead to a conformation that forms an active center, while an antagonist may stabilize a conformation that either does not bring Asp, His, Ser into proper orientation or has the correct conformation but is missing a required residue. Whether transduction involves a serine protease-like autolysis of the glucagon-bound receptor complex to release a 33-kDa or smaller active fragment that activates the G protein remains to be proven using pure receptor protein.

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