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Effects of Iodination of Tyrosyl Residues on the Binding and Action of Glucagon at Its Receptor†

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ABSTRACT: The binding and action of glucagon at its receptor in hepatic plasma membranes have been compared, as a function of pH, with that of glucagon containing iodotyrosyl residues. Iodinated glucagon, at pH 7.0 and below, binds to the receptor and activates adenylate cyclase with an affinity about threefold higher than that of native glucagon. At pH 8.5, the affinity of the receptor for native glucagon is the same as that seen at pH 7.0. However, iodinated glucagon binds with a lowered affinity with increasing pH. The decreased affinity of the iodinated hormone correlates with ionization of the iodotyrosyl phenoxy group, which has a pK_a of 8.2. It is suggested that the decreased affinity is actually due to the inability of the ionized iodoglucagon to bind to the receptor. The relative potency of native and iodoglucagon will depend, therefore, on the concentrations of ionized and un-ionized species of iodoglucagon, which in turn depend on the pH of the medium. We conclude that incorporation of iodine atoms in the tyrosyl residues of glucagon has two major effects: (i) the iodine atom increases hydrophobic interaction of the hormone with the receptor and (ii) ionization of the phenoxy groups results in the loss of biological activity possibly as the result of loss of hydrogen bonding capability. Thus, the tyrosyl residues in glucagon are critically involved in the function of the hormone.

There are conflicting reports in the literature concerning the effect of iodination of glucagon on its binding and action on receptor-mediated adenylate cyclase systems. While enhanced biological potency has been reported for iodoglucagon (Bromer et al., 1973; Desbuquois, 1975), in two separate studies we were unable to detect any difference in the potency of native and monoiodoglucagon (Rodbell et al., 1971; Lin et al., 1975). The reasons for this discrepancy need to be clarified since resolution might yield a better insight into the general problems of utilizing iodinated hormones for investigating the binding and action of hormones at their receptors.

Iodine is principally and equally incorporated into the two tyrosyl residues of glucagon (Desbuquois, 1975). Iodination of tyrosyl residues produces two opposing effects: (i) an increase in the hydrophobicity of the phenoxy ring, and (ii) a reduction in the pK_a for hydroxyl group of tyrosine. A major effect due to iodination of peptide hormones can be expected if the iodinated tyrosyl residues are critically involved in the function of these molecules. We considered the possibility that these two effects resulting from iodine incorporation may alter the binding and action of the iodinated glucagon on the hepatic adenylate cyclase system. By studying the binding and action of iodoglucagon at a pH where the phenoxy group is not ionized, the effect of increased hydrophobicity can be examined. In this report, we provide evidence that ionization of the iodinated tyrosyl residues results in a marked decrease in the binding of glucagon, whereas the un-ionized species displays both an increased affinity for the glucagon receptor and increased potency in the activation of adenylate cyclase. Studies to be reported elsewhere[1] will deal with the binding kinetics of unlabeled native glucagon.

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[1] C. Londos, manuscript in preparation.

Experimental Section

Materials. [α-^{32}P]ATP was obtained from International Chemical and Nuclear. [^{3}H]cAMP[2] was from New England Nuclear. ATP, cAMP, creatine phosphate, creatine phosphokinase, and dithiothreitol were purchased from Sigma. Crystalline glucagon was purchased from Eli Lilly and Co. Oxoid Nuflow Membrane filters and Na^{125}I (400 mCi/ml) were from Amersham/Searle, and Chloramine-T was from Eastman Kodak. Talc tablets were from Ormont Drug and Chemical Co., Englewood, N.J. Bovine serum albumin (fraction V) was from Miles.

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 nitrogen.

Preparations of [^{125}I]Glucagon. [^{125}I]Glucagon was prepared by a modification of the procedures previously described (Rodbell et al., 1971; Giorgio et al., 1974). Glucagon (2 nmol), Na^{125}I (2 mCi, about 1 nmol), and Chloramine-T (44 nmol) were mixed in a final volume of 50 μl of 0.5 M phosphate buffer, pH 7.0. After 15 s at 25 °C, the reaction was stopped by adding 50 μl of sodium metabisulfite (500 nmol) and 100 μl of NaI (10 μmol). Iodinated hormone was adsorbed to talc; after washing and extraction with 50% ethanol, [^{125}I]glucagon was finally dissolved in 2 ml of 1% bovine serum albumin in Tris-HCl buffer 20 mM, pH 7.5, and the solution was cleared by Millipore filtration. [^{125}I]Glucagon was further purified on a small cellulose column (0.6 × 2 cm) equilibrated with 1% bovine serum albumin in Tris-HCl (20 mM), pH 7.5. The column was eluted in succession with 4 ml of 1% bovine serum albumin, 6 ml of water, and 0.4 ml of 50% methanol; [^{125}I]glucagon was eluted in the subsequent 0.5 ml of 50% ethanol. The specific activity was about 10^6 cpm/pmol of glucagon (about 0.3 ^{125}I atom per molecule).[3] The concentration of biologically active glucagon was estimated from its ability to activate hepatic adenylate cyclase.

Preparation of Iodoglucagon. The reaction was performed as described for [^{125}I]glucagon, except that 120 nmol of glucagon, 250 nmol of NaI, and 1.1 μmol of Chloramine-T were used in 2.5 ml of 0.5 M phosphate buffer, pH 7.0. The reaction was allowed to proceed for 30 s at 30 °C. Na^{125}I (5 μCi) was added as a tracer to follow the recovery of product and to determine the extent of iodination; the trace amount of radioactivity did not interfere with the subsequent studies. Iodoglucagon was desalted on a Sephadex G-25 column (1.5 × 28 cm) using 5% acetic acid as eluent. The product contained 1.2 iodine atoms per molecule of glucagon. Amino acid analysis indicated only 10% loss of tryptophan, while all other amino acids remain intact, when compared with analysis of native glucagon.

Assay for Glucagon Binding. Binding of [^{125}I]glucagon was performed as described previously (Lin et al., 1975). The radioactivity retained on the filter in the presence of 10^{-6} M native glucagon, was taken as nonspecific binding. The net binding was obtained by subtracting nonspecific binding from the total. The concentrations of [^{125}I]glucagon, glucagon, and iodoglucagon, as well as the pH values (Tris-acetate buffer, 50 mM), are shown in the legends to the figures.

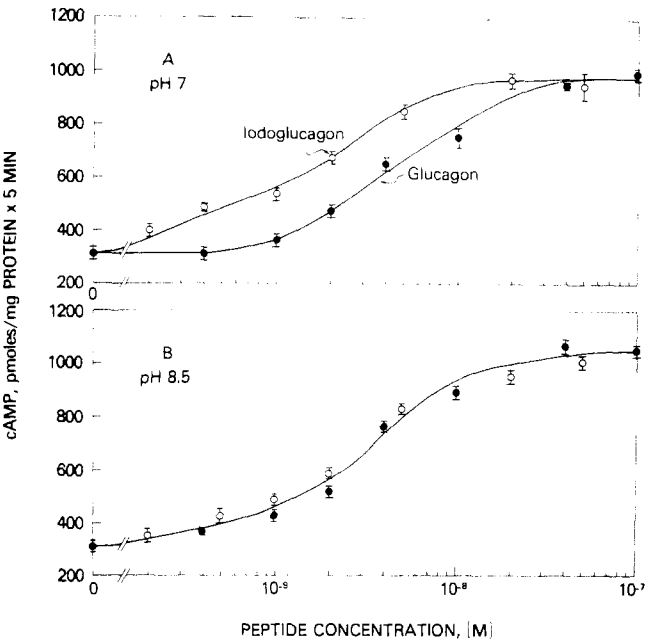

FIGURE 1: Activation of hepatic adenylate cyclase by native (●—●) and iodoglucagon (○—○). The activity of adenylate cyclase was measured at pH 7 (panel A) and pH 8.5 (panel B) as described in the Experimental Section. Bars represent standard deviation of four determinations.

Assay for Adenylate Cyclase Activity. The assay was carried out as described (Lin et al., 1975; Salomon et al., 1974), except that ATP at 1 mM, $MgCl_2$ (10 mM), and GTP (5 μM) were used. Glucagon or iodoglucagon, dissolved in Tris-HCl buffer (20 mM), pH 7.5, containing 1 mg/ml of bovine serum albumin was added at the concentrations shown in the figures. Fresh solutions were prepared for each experiment.

Analytical Methods. Protein concentration was determined according to Lowry et al. (1951), with bovine serum albumin as standard. The amino acid content of iodoglucagon was determined on a JEOL JLC-6AM analyzer after hydrolysis with methanesulfonic acid (Liu, 1972). No correction was made for amino acid destruction during hydrolysis. Concentrations of both glucagon and iodoglucagon were determined by amino acid analysis and by a ninhydrin method (Fruchter and Crestfield, 1965; Moore, 1968).

Results

Activation of Adenylate Cyclase by Glucagon and Iodoglucagon as a Function of pH. In previous studies we found that, at pH 7.6, the potency of glucagon and monoiodoglucagon was indistinguishable as activators of adenylate cyclase in hepatic membranes (Rodbell et al., 1971; Lin et al., 1975). As shown here (Figure 1B), native hormone and iodoglucagon are equally effective also at pH 8.5. Figure 1A shows, however, that iodoglucagon is a more potent activator than glucagon at pH 7.0 with an apparent K_m (half-maximal activation concentration) that is approximately threefold lower than that given by glucagon which was identical at pH 7 and 8.5. These findings suggest that the difference in potency of iodoglucagon and native glucagon is related to an effect of pH on the structure of iodoglucagon rather than on the receptor component

[2] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane.

[3] One of the major difficulties encountered in the study of [^{125}I]glucagon binding is the continuous damage of glucagon due to radiolysis during storage. We have evidence that small fragments of radioactive material are produced from cleavage of glucagon after only 2 weeks of storage. Therefore, not all of the radioactivity is bindable and an accurate estimation of specific activity thus becomes very difficult. While we have given values for specific activity and amount of glucagon in this study, they can only be considered as approximate values. However, precaution has been taken that [^{125}I]glucagon was used only for 10 days from the day of preparation.

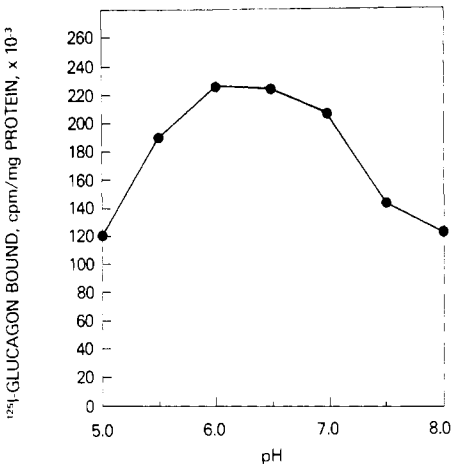

FIGURE 2: pH dependency of [^{125}I]glucagon binding. The binding assays were carried out with 4×10^{-10} M [^{125}I]glucagon (specific activity, about 10^6 cpm/pmol) as described in the Experimental Section.

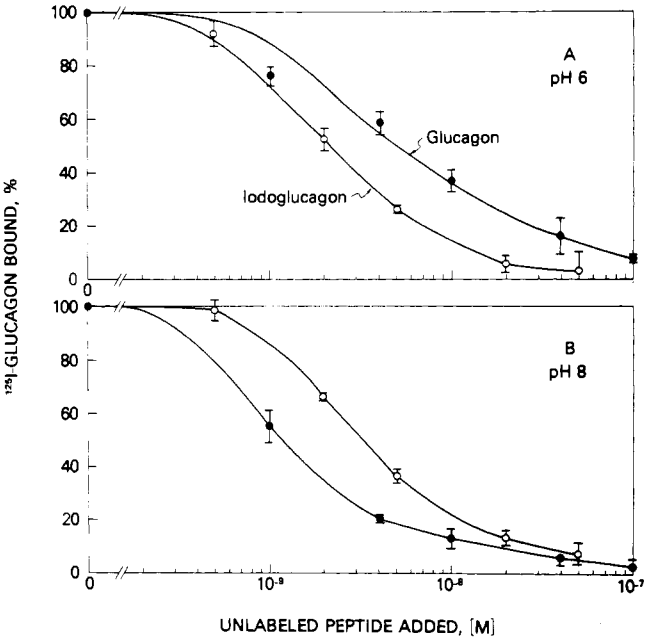

FIGURE 3: Effects of dilution of [^{125}I]glucagon with unlabeled native glucagon (●—●) or iodoglucagon (○—○). Binding assays were carried out with 4×10^{-10} M [^{125}I]glucagon (specific activity, about 10^6 cpm/pmol) and the indicated concentratiions of unlabeled peptide at pH 6 (panel A) or pH 8 (panel B) as described in the Experimental Section. Bars represent standard deviation of four determinations. The [^{125}I]glucagon bound in absence of added unlabeled peptide, taken as 100%, was 1.8×10^5 cpm/mg at pH 6.0 and 5.3×10^4 cpm/mg protein at pH 8.0.

involved in activation of adenylate cyclase. This assumption was further tested by examining the effect of pH on the binding of glucagon and the iodinated derivative to the receptor.

pH Dependence of [^{125}I]Glucagon Binding. When the pH profile of [^{125}I]glucagon binding to hepatic membranes was examined, it was found that binding of the labeled hormone reached a peak at pH 6.0 and declined to about 50% of this maximal value at pH 7.5 and above (Figure 2).

In order to compare the relative affinities of iodoglucagon and native glucagon for the receptor as a function of pH, the ability of the two forms of the hormone to compete with the binding of [^{125}I]glucagon was studied at pH 6.0 and 8.0. As shown in Figure 3, iodoglucagon competed more effectively than native glucagon at pH 6.0 (panel A) whereas, at pH 8.0 (panel B), the situation was reversed. These findings provided evidence that iodoglucagon has a higher affinity for the receptor sites than native glucagon as the pH is decreased and are consistent with the higher affinity displayed by iodoglucagon activation of adenylate cyclase as a function of decreasing pH (Figure 1).

The change in the affinity of iodoglucagon for the receptor as a function of pH was estimated from double-reciprocal plots of similar binding data (not shown). Half-maximal occupation of the receptors occurred with 2.5 nM iodoglucagon at pH 6 and with 10 nM iodoglucagon at pH 8. This difference is comparable with the estimated threefold difference in the half-maximal concentrations required for activation of adenylate cyclase by iodoglucagon examined at pH 7.0 and pH 8.5 (Figure 1). The interpretation of affinity at pH 8 derived in this manner is difficult, however, since at this pH, iodoglucagon probably exists as mixtures of ionized and un-ionized forms.

Discussion

As first shown by Bromer et al. (1973) and subsequently by Desbuquois (1975) in a more detailed study, incorporation of iodine atoms in the tyrosyl residues of glucagon results in increased potency of the iodinated hormone on adenylate cyclase activity and increased binding to the glucagon receptors in hepatic membranes. The present study agrees with their findings. However, these differences between native and iodoglucagon depended on the pH of the incubation medium. With increasing pH, the quantity of iodoglucagon bound decreased and the concentration giving half-maximal activation became equivalent to that of native glucagon. By contrast, glucagon action did not show such marked pH dependency (cf. Figure 1). Since one of the effects of increasing pH on iodoglucagon is to increase ionization of the phenoxy groups, it seems likely that the decreased binding is due to the ionization of these groups. The pK_a of monoiodinated tyrosine is about 8.2. Assuming that the same pK_a applies to the iodinated tyrosyl residues, then nearly 70% of the tyrosyl residues in glucagon would be ionized at pH 8.5. However, iodinated glucagon may be a heterogeneous mixture of monoiodo, diiodo (on a single tyrosyl residue), and one iodine atom on each of the two residues, each of which may have differing structures and, therefore, interactions and potency at the glucagon receptor.

The following possibilities can be considered to account for the decreased affinity of iodoglucagon with increasing pH and the finding that iodoglucagon and native glucagon become equivalent in biological potency even though the iodinated species appears to bind with lower affinity than native glucagon. One possibility is that the ionized species do not bind to the receptor. Correcting for the concentration of un-ionized iodoglucagon estimated at pH 8.5, thus, would result in a reduction in concentration of active species by about 70%. But with three times the potency (or affinity) of un-ionized iodoglucagon relative to native glucagon, the net effect on activity would be equipotent effects of iodoglucagon and native glucagon on adenylate cyclase activity, as was observed (cf. Figure 1B). A second possibility is that the ionized species of iodoglucagon bind to the receptor with much lower affinities than the un-ionized species. In this case, the binding and activity curves would reflect the average binding of the heterogeneous population of species. This possibility is obviously more difficult to evaluate.

We favor the first possibility both for its simplicity of in-

terpretation and the reasonable agreement with the data. In either case, studies with iodoglucagon provide good evidence for the following: (i) the tyrosyl residues play a crucial role in the binding and action of glucagon, in accord with theoretical considerations (Chou and Fasman, 1975) and the x-ray crystallographic analysis (Sasaki et al., 1975); (ii) ionization of the phenoxy groups appears to have important deleterious effects on the structure required for the binding and action of the hormone, possibly through loss of hydrogen binding; and (iii) incorporation of an iodine atom modifies the nature of glucagon binding and action to give a more potent analogue.

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HeLa Cell DNA Polymerase γ: Further Purification and Properties of the Enzyme†

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ABSTRACT: DNA polymerase γ has been purified over 60 000-fold from HeLa cells which contain no detectable type C viral particles. This purified enzyme shows a specific activity of 25 000 units/mg of protein which is comparable to the known specific activity of homogeneous preparations of human α and β polymerases. The isolated enzyme shows apparent molecular weights ranging from 160 000 to 330 000 according to the method of analysis. The enzyme exhibits optimal activity for copying poly(A) in the presence of 50 mM KPO_4 and 130 mM KCl and, under these conditions, copies poly(A) 20 times more rapidly than activated DNA. These assay conditions permit a clear distinction between the γ-polymerase and DNA polymerase β which is markedly inhibited by phosphate at this concentration. A comparison of the copying of activated DNA, poly(dA) and poly(A) by DNA polymerases α, β, and γ under optimal assay conditions for each enzyme is presented. Studies with synthetic and natural nucleic acid templates also show the γ-polymerase to behave differently than the reverse transcriptases of avian myeloblastosis virus or Rauscher leukemia virus.

There are now four presumptive DNA polymerases in mammalian cells (Weissbach, 1975). Three of the enzymes, DNA polymerases α, β, γ, are presumed to be involved in the replication or repair of nuclear DNA, whereas the mitochondrial DNA polymerase is associated with the organelle and is assumed to participate in mitochondrial DNA synthesis. The γ-polymerase is relatively efficient in its use of synthetic RNA templates such as poly(A), a property not unlike that shown by the reverse transcriptases of the type C tumor viruses. Thus, one of the objectives of this study was to obtain highly purified preparations of DNA polymerase γ in order to compare the cellular enzyme to the viral enzyme.

We have now purified human DNA polymerase γ more than 60 000-fold from HeLa cells which are free of demonstrable oncorna virus particles. The optimal conditions needed for the copying of various synthetic and natural DNA and synthetic RNA templates by this enzyme preparation have been studied. These studies have provided a clearer picture of the differences between DNA polymerases α, β, and γ, and the reverse transcriptase of oncorna viruses, but have not enabled us to detect the copying of natural RNA by the HeLa cell γ-polymerase.

Materials and Methods

[^{3}H]Deoxynucleoside triphosphates were purchased from Schwarz/Mann, Orangeburg, N.Y. The oligonucleotides $(dA)_{12-18}$, $(dC)_{12-18}$, and $(dG)_{12-18}$ were obtained from Collaborative Research, Inc., Waltham, Mass.; $(dT)_{12-18}$ was supplied by Schwarz/Mann. $(A)_{12-18}$ was prepared from reovirus RNA acccrding to Stoltzfus and Banerjee (1972). Poly(A), poly(dA), poly(C), poly(dC), poly(I), poly(dT), and

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