Preparation and Characterization of a Fully Iodinated Insulin Derivative*

LISE GRUEN,† MICHAEL LASKOWSKI, JR.,‡ AND HAROLD A. SCHERAGA

From the Department of Chemistry, Cornell University, Ithaca, New York

(Received for publication, March 10, 1959)

In the course of a study of the thermodynamics of the ionization of the lysyl residue of insulin (1), it was found desirable to make an iodinated derivative of insulin in order to correct the four tyrosyl residues in the monomer (molecular weight 5733 (2)) to 3,5-diiodotyrosyl groups which have lower pK values (3). Numerous properties of the iodinated derivative have been studied in order to ascertain whether the iodination of insulin was specific for the tyrosyl residues or whether other changes occurred in the molecule.

EXPERIMENTAL

Preparation of Iodinated Insulin

It was necessary to choose a procedure which would completely iodinate the tyrosyl residues in insulin without causing side effects. Optimal conditions for the specific iodination of tyrosyl residues in proteins have been discussed by Hughes and Straussle (4). Harington and Neuberger (5) have prepared a fully iodinated insulin derivative, and Oster and Malament (6) have prepared and crystallized iodinated insulin derivatives which were up to 91 per cent iodinated on the tyrosyl residues. A slight modification of the procedure of Oster and Malament (6) has been followed in the present work. In order to facilitate iodine analyses, radioactive 131I tracer has been added to the iodinating reagent.

The 131I tracer was obtained from Oak Ridge National Laboratory. It was at a concentration of about 16 mc. per ml. in sodium sulfite solution and had a radiochemical purity greater than 99 per cent. To check for the presence of long lived radioactive impurities which could lead to errors in the analyses, standard solutions containing 131I were counted daily for 4 weeks, and no deviation from the 0-day half-life could be observed. The counter was a well type scintillation counter with a sodium iodide crystal.

Iodinating Reagent—The iodinating reagent was prepared from a 0.5 m potassium iodide solution. An accurately weighed sample of approximately 2.25 gm. of potassium iodide (Malinkrodt A.R.) was dissolved in water in a 25 ml. volumetric flask, approximately 1 mc. (50 ~1. of the Oak Ridge solution) of 131I tracer was added, the solution was brought to 25 ml. with distilled water, and was shaken well. Samples, 10, 25, and 75 ~1. of the labeled potassium iodide solution were transferred to 1-oz. screw cap vials, with the use of micropipets. These were set aside for use as standards in counting, since they contained known total amounts of iodide labeled with tracer. The remainder of the potassium iodide solution was converted into the triiodide reagent used in the iodination by adding 3 ml. of 2 N sulfuric acid and 2 ml. of 3 per cent hydrogen peroxide and stirring for 4 hours. This produced a solution which was approximately 0.15 n in iodide and contained iodide and iodine in the ratio of 4 I⁻ per I⁻.

Procedure—All the insulin used in this work was Lilly crystalline beef zinc insulin, Lot No. 535664. A 500-mg. sample of zinc insulin was dissolved in 50 ml. of 0.5 m glycine buffer which had been adjusted to pH 9.5 at 0° with potassium hydroxide. The solution was cooled to 0° and kept (stirred) in an ice bath. The triiodide solution was added dropwise from a buret over a period of about 3 hours, until a slight excess of iodine had been added (approximately 9 ml.). The solution, which was then yellow, was kept cold and stirred 1 hour longer. It was then dialyzed for 1 week at 5° against numerous changes of distilled water, in order to ensure complete removal of excess iodine and glycine buffer. On the 3rd day of dialysis, the iodinated insulin was usually dialyzed against 0.002 n potassium hydroxide for 2 to 3 hours and then against distilled water again. This procedure seemed to improve the solubility of the final product. After dialysis, a sample was taken for iodine analysis and the remainder of the product was lyophilized and stored below 0°. The over-all yields of iodinated insulin were from 80 to 90 per cent of the theoretical.

Iodine Analysis—A known volume, 1 to 3 ml., of the aqueous solution of iodinated insulin to be analyzed was placed in a screw cap vial and counted on the scintillation counter. A labeled potassium iodide standard of approximately the same level of radioactivity was brought to the same volume with water and counted in the same manner as the unknown. Since the ratio of radioactive iodine to total iodine is the same in both the standard and the sample being analyzed, and since the total iodine in the standard is known, the iodine content of the insulin solution is readily determinable from the ratio of the counts in...
the sample and standard, after correction for background radiation. The protein concentration was determined by heating a known volume of the solution to constant weight in an oven at 104°. It was found that iodine analyses performed on lyophilized material which had been heated at 104° and then dissolved in water gave results identical, within the experimental error, with analyses on the dialyzed protein solution before freeze-drying. Hence, no iodine was lost on lyophilization or on heating at 104°.

RESULTS AND DISCUSSION

Iodine Content—All the iodinated insulin analyzed, which had been made with several different preparations of labeled iodine, was found to be 94 to 100 per cent iodinated, on the basis of two iodine atoms per tyrosyl residue (or eight iodine atoms in each insulin monomer). Therefore, all the iodinated insulin prepared can be assumed to be 100 per cent iodinated within the experimental error (about 5 per cent) in the analyses, and a molecular weight of 6740 has been assumed for the iodinated monomer throughout this work. It should be noted that all the iodine incorporated into the protein may not have gone onto the tyrosyl residues, but this is unlikely in view of the excellent agreement in stoichiometry, and the titration behavior and other properties of the iodinated derivative to be discussed below.

The lyophilized iodinated insulin is cream-colored. It is soluble in 0.3 M KCl to the extent of 1 per cent or more at pH values above 7.5, and is insoluble in acidic salt solutions down to pH 0 or less. In lower concentrations (about 0.03 per cent) it remains soluble down to about pH 6.8, and again becomes soluble below pH 4 in the absence of salt. Three solubilities are in agreement with those found by Harington and Neuberger (5) for their completely iodinated insulin. The zinc content of various preparations of iodinated insulin, as determined by the disodium ethylenediaminetetraacetate titration method described by Flaschka (7), ranged from 0.2 to 0.7 mole of zinc per mole of dimer (with most samples containing between 0.4 and 0.6 mole of zinc per mole of dimer). This can be compared with the native crystalline insulin which was found to contain 1 mole of zinc per mole of dimer, in agreement with the manufacturer’s analysis.

Paper Electrophoresis and Sedimentation—Paper electrophoresis of the iodinated derivative, carried out at room temperature at 4 ma. and 60 volts for 17 hours in a Spinco cell of the Durrum type (model R, series C) using Spinco 0.075 M barbiturate buffer, pH 8.6, produced only a single electrophoretic component when stained with ninhydrin and with Pauly reagent. It traveled faster than a native insulin control on another strip, as would be expected if the tyrosyl residues had been converted to 3,5-diiodotyrosyls, because the latter would be more ionized at pH 8.6, giving the iodinated insulin a larger net charge than the native. Sedimentation patterns of a 1 per cent iodinated insulin solution at pH 10 and of a 1 per cent iodinated insulin solution left at pH 12 for 1.5 hours and then brought to pH 10 were obtained with the use of a synthetic boundary cell in the Spinco model E ultracentrifuge at 59,780 r.p.m. Each sample showed only a single peak with $s_{20,w}$ equal to approximately 1.8 Svedberg units, as estimated from the peak displacements. The zinc content of these samples was 0.6 mole of zinc per mole of dimer. A study (8) of 1.5 per cent zinc insulin in the same cell gave $s_{20,w}$ equal to 2.19 S, corresponding to a molecular weight of approximately 18,000 (calculated by using an approximate diffusion coefficient obtained from the sedimentation patterns). Frederick’s measurements (9) on 0.25 per cent insulin solutions in a synthetic boundary cell gave sedimentation constants between 1 and 3.5 S, depending on the zinc content (between 0 and 1 mole of zinc per 12,000 gm. of protein). The electrophoresis and sedimentation results indicate that the iodinated insulin is homogeneous and is not broken down by iodination or by exposure to pH 12.

End Group Analysis and Chromatography—A DNP end group analysis was carried out on the iodinated insulin, following the procedure described by Fraenkel-Conrat et al. (10). An ascending chromatogram of the ether extract, with the use of a toluene-pyridine-2-chloroethanol solvent, showed only two spots, corresponding to DNP-glycine and DNP-phenylalanine, the same end groups as in native insulin. Hence, it is unlikely that any peptide bonds were hydrolyzed during the preparation of the iodinated derivative. The $\epsilon$-DNP-lysine, O-DNP-tyrosine, and imidazole-substituted DNP-histidine are soluble in the aqueous phase, and therefore would not appear on the chromatogram of the ether extract. A paper chromatogram of the acid hydrolyzate of iodinated insulin, with the use of a butanol-acetic acid-water solvent (11), showed all the amino acids of native insulin, including tyrosine. Since acid hydrolysis has been found to remove iodine from iodoproteins (12), the absence of diiodotyrosine on the chromatogram cannot be considered to be evidence against the complete iodination of the tyrosyl residues of insulin.

Ultraviolet Absorption Spectra—The ultraviolet absorption spectrum of the iodinated insulin derivative is compared with that of 3,5-diido-L-tyrosine in Fig. 1. The iodinated insulin was a 0.01 per cent solution in 0.01 M KOH and had a pH of 11.88; the 3,5-diido-L-tyrosine (Mann Research Laboratories, Inc.) was a 0.006 per cent solution in 0.01 M KOH and had a pH of 12.2. Concentrations were determined by using quantitative dilutions of solutions prepared from directly weighed samples whose weights had been corrected for moisture content as deter-

\[\text{Molar Extinction Coefficient} \times 10^4\]

\[\begin{array}{c}
\text{Wavelength (\text{m\mu})} \\
260 & 280 & 300 & 320 \\
0.10 & 0.15 & 0.20 & 0.25 \\
0.70 & 0.75 & 0.80 & 0.85 \\
\end{array}\]

\[\Delta \text{Diiodotyrosine; pH 12.2} \quad \bullet \text{Iodinated Insulin} \times \frac{1}{4}; \text{pH 11.88}\]

Downloaded from http://www.jbc.org/ by guest on August 27, 2017

\[3\] The abbreviation used is: DNP, 2,4-dinitrophenyl.
The measurements were made on a Beckman model DU spectrophotometer with photomultiplier, using 1-cm. quartz cells. The similarity of the spectra shown in Fig. 1 supports the premise that the tyrosyl residues in insulin have been converted to diiodotyrosyl. The diiodotyrosine spectrum has a maximum at 311 μm with a molar extinction coefficient of 5.80 × 10^3, in good agreement with Oster and Malament's value (6) of 5.815 × 10^3 at 311 μm and also with the values determined by Herriott (13) and by Gemmill (14). The iodinated insulin spectrum has a maximum between 311 and 312 μm with an extinction coefficient of 5.35 × 10^3 per mole of monomer, which is about 10 per cent lower than the molar extinction coefficient for diiodotyrosine. The iodinated insulin curve also appears to be shifted somewhat to longer wave lengths. Oster and Malament (6) reported a slight depression in the ultraviolet absorptivity of the 3,5-diiodotyrosyl residues in iodinated insulin, but part of their result may be due to the incomplete ionization of the diiodotyrosyl groups, since their measurements were made at pH 9.74. An attempt to more nearly reproduce the iodinated insulin spectrum by including small

Fig. 2. Spectrophotometric titrations of 3,5-diiodo-L-tyrosine, Δ, at 312 μm and of iodinated insulin in 0.3 M KCl, □, and in water, ○, at 311 μm. As in Fig. 1, the molar extinction of iodinated insulin has been divided by four. The curve drawn through the points for diiodotyrosine, Δ, is a theoretical titration curve for a group with pK of 6.5.

Fig. 3. Titration curves at 25° of zinc-free insulin, □, and of iodinated insulin, ○, in 0.3 M KCl, showing the difference, Δr, in the number of groups titrated in the region of diiodotyrosyl and tyrosyl ionization. The zero for the r-scale is arbitrary. The protein was partially insoluble in the region to the left of the vertical bar on each curve.
amounts of the spectra of tyrosine and moniodotyrosine (13) in that of diiodotyrosine (assuming additivity of the spectra) proved unsuccessful. From the shape of the absorption spectrum it appears, therefore, that the slight reduction in the absorptivity of the diiodotyrosyl residues in iodinated insulin is most probably due to their incorporation into the protein, rather than to the incomplete iodination of the tyrosyl residues or to the formation of some moniodotyrosine.

Spectrophotometric Titrations—The spectrophotometric titration curve at 312 μ of 3,5-diiodo-tyrosine and those at 311 μ of iodinated insulin in water and in 0.3 M KCl, determined following the method first described by Stenström and Goldsmith (15), are shown in Fig. 2. The spectral measurements were made as above. The pH values were determined immediately before and after the spectrophotometric measurements on the A scale of a Beckman model GS pH meter with external glass and calomel electrodes. All solutions were unbuffered and carbon dioxide was not excluded. The apparent pK for 3,5-diiodotyrosine was found to be 6.5, in reasonable agreement with the value of 6.42 obtained by Crammer and Neuberger (3), and also with the value of 6.48 obtained by Dalton et al. (16) with a direct titration method. The apparent pK values for the iodinated insulin titrations are more uncertain because of the spreading of the titration curves and the absence of points in the insolubility region. The apparent pK values of the phenolic hydroxyl groups of iodinated insulin in water and in 0.3 M KCl are 7.9 and 7.2, respectively, as determined from the mid-points of the curves (assuming that the low pH limiting value of the extinction coefficient of iodinated insulin is the same in 0.3 M KCl as in water). These apparent pK values are quite consistent with the presence of 3,5-diiodotyrosyl groups in the iodinated derivative, since electrostatic interactions would be expected to increase the apparent pK as the protein becomes negatively charged.

Electrometric Titrations—Titrations of 0.001 M zinc-free insulin and of 0.002 M iodinated insulin in 0.3 M KCl at 25°C were carried out between pH 7.5 and pH 12 with 1 N KOH. The pH values were read on the A scale of the model GS pH meter. The calculation of the titration curve has been described elsewhere (1). The results are shown in Fig. 3, in which τ, the moles of hydrogen ion dissociated per mole of protein, is plotted as a function of pH. The curve for zinc-free insulin agrees with that of Tanford and Epstein (17) for zinc-free insulin in 0.075 M KCl, to within better than one-half group over the pH range shown.

The two curves in Fig. 3 were matched on the r-scale, at the high pH end where all the phenolic hydroxyl groups are presumably titrated. Because of the scatter in the points in this region and because of the possible effect of iodination on the titration of groups other than the phenolic, e.g. through changes in electrostatic interactions, the matching of the two titration curves may be off by as much as one group. However, it is apparent that several groups which ionize between pH 8 and pH 11 in zinc-free insulin have lower pK values in the iodinated insulin, as would be expected if the tyrosyl residues have been converted to diiodotyrosyls. The maximal difference in the number of groups titrated along the two curves is slightly less than four, the number of tyrosyl residues in each insulin monomer. However, if there is any overlap between the regions of ionization of tyrosyl and diiodotyrosyl groups, the difference between the two curves would be less than four even if all the tyrosyls are fully iodinated in the iodinated derivative. It should be noted that a difference of up to one group between the two curves may be caused by a difference in zinc content of the two samples. Each zinc forming complexes to the protein produces two extra titratable groups with an intrinsic pK around 8.0 (17); the iodinated insulin may have contained up to ½ mole of zinc per mole of protein (probably less), whereas the zinc-free insulin contained none.

CONCLUSION

The properties of the iodinated insulin derivative indicate that the four tyrosyl residues have been completely iodinated to 3,5-diiodotyrosyl groups with no other apparent modification of the protein molecule. As a result of the iodination of the protein, the overlapping of the region of ionization of the lysyl group with that of the phenolic hydroxyl groups has been considerably reduced. The thermodynamics of the ionization of the lysyl group have been reported elsewhere (1).

SUMMARY

An iodinated derivative of insulin containing eight atoms of iodine in each monomer unit has been prepared.

The properties of the iodinated insulin, as determined by sedimentation in the ultracentrifuge, paper electrophoresis, DNP end group analysis, paper chromatography, ultraviolet absorption spectra, and spectrophotometric and electrometric titration curves, support the conclusion that all the iodine has been substituted on the four tyrosyl residues in the insulin monomer, and that no other apparent modifications in the protein molecule have taken place.

REFERENCES
