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Biotinylinsulins as potential tools for receptor studies

(peptide hormones/insulin/avidin–biotin complex/insulin receptors/affinity chromatography)

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ABSTRACT **The preparation of affinity columns that contain insulin attached to Sepharose in a targeted manner by way of biotin–avidin noncovalent bonds is described. Insulin was acylated selectively at the amino terminus of the B chain with the *N*-hydroxysuccinimido ester of biotin to form $N^{\alpha,B1}$-biotinylinsulin. The ability of this modified insulin to stimulate rat epididymal adipocytes was (mean ± SD) 94 ± 9.6% (P, 0.05) that of the control insulin. $N^{\alpha,B1}$-Biotinylinsulin displaced 4-hydroxyazobenzene-2′-carboxylic acid from avidin, demonstrating affinity for this protein. The formation of the $N^{\alpha,B1}$-biotinylinsulin–avidin complex was visualized by cellulose acetate electrophoresis at pH 4. $N^{\alpha,B1}$-Biotinylinsulin combined with avidin attached to Sepharose to form affinity columns in which the hormone was attached to the support by strong noncovalent bonds. The determination of the loading of avidin–Sepharose columns with biotinylinsulin was greatly facilitated by the attached biotin which provided a marker whose concentration could be assessed accurately by titration with avidin. Biotinylinsulin attached to avidin–Sepharose beads retained the ability to stimulate rat epididymal adipocytes. The activity of several samples of these beads was about 15% that of free biotinylinsulin, based on the amount of biotinylinsulin anchored to the support. The advantages of biotinylated hormones for the targeted attachment of hormones to solid supports are discussed.**

The effectiveness of affinity columns for receptor studies is dependent on the highly specific complementarity between hormone and receptor, and consequently the binding region of the hormone should not be modified. The problem of attaching polyfunctional molecules such as insulin to solid supports without disturbing their receptor-binding potential represents a challenging problem which we address in this communication.

Insulin was selectively acylated with biotin (Fig. 1 *upper*) at the amino terminus of the B chain to form $N^{\alpha,B1}$-biotinylinsulin (Fig. 1 *lower*). This compound, named "biotinylinsulin" for simplicity's sake, was prepared with the expectation that it would exhibit affinity for insulin receptors and also toward the egg-white protein, avidin (for a review, see ref. 1). As a result of this bivalency, it provides the basis for the targeted preparation of selective insulin receptor affinity columns. Such columns result when the biotinylinsulin is added to a solid support containing covalently attached avidin. Fundamentally they are based on the high affinity of biotin for avidin (dissociation constant, K_D, approximately 10^{-15}).

The advantages of the method are: (*i*) biotin can be attached to the hormone in a targeted manner; (*ii*) the chemical manipulations are performed with the free hormone, and thus their effect on binding capacity and biological activity can be readily assessed by bioassay; (*iii*) the attachment of the modified hormone to the support involves a strong noncovalent bond that forms specifically and spontaneously on simple mixing of the components; and (*iv*) the operations involved in preparing columns of this type lend themselves to scaling up. The design of affinity columns delineated in this and a previous communication (2) differs significantly from established approaches and appears to be broadly applicable to other systems.

Abbreviation: Boc, tertiary butoxycarbonyl.

EXPERIMENTAL

Materials. Insulin (bovine crystalline, 25 units/mg) was obtained from the firm Dr. Ch. Brunnengräber, Lübeck, and was also isolated from insulin C component generously supplied by Eli Lilly and Co., Indianapolis. Avidin was from Worthington Biochemical Corp.; 4-hydroxyazobenzene-2′-carboxylic acid was from Eastman Kodak, and Ponceau S from E. Merck and Co., Darmstadt. Cellulose acetate strips were from Schleicher and Schüll, Sephadex gels were from Pharmacia Fine Chemicals, and DEAE-cellulose (DE-52) was from Whatman. The (+)biotin was a gift from Hoffmann-La Roche, Inc., Nutley, NJ.

General Procedures. Column eluates were monitored with LKB I or II instruments. Cellulose acetate electrophoresis was performed in 2.4 M formic acid/4 M urea, pH 2.2, buffer (3) except where noted. Samples (approximately 0.4 mg) in approximately 20 μl of buffer were applied to the cellulose acetate strips. After 3–4 h at 15 V/cm, the strips were stained with a solution of 0.2% (wt/vol) Ponceau S in 3% (wt/vol) trichloroacetic acid (4, 5). Electropherograms were evaluated at 435 nm with a Zeiss PM QII spectrophotometer equipped with a chromatogram scanner, using the staining factors given in ref. 5. To obtain the results shown in Fig. 2, avidin (0.125 μmol/ml), biotinylinsulin (0.5 μmol/ml), and mixtures of the two in the proportions indicated were applied (10 μl samples), in pH 2.1, 2.4 M sodium formate buffer containing 0.1 M urea, to cellulose acetate strips. Electrophoresis was performed in 1.0 M acetate buffer (adjusted to pH 4.0 with pyridine) containing 0.1 M urea. After 2.5 hr at 15 V/cm, the strips were stained with Ponceau S and the electropherograms were evaluated as described above.

The biotin content of biotinylinsulin was determined according to Green (6). Sulfitolysis was performed with 1 mg of material essentially as described (7). The reaction mixtures were applied directly to paper strips for electrophoresis in the pH 2.2 buffer at 4 V/cm.

Ion exchange chromatography was performed at 4°. For preparation of the Tris/urea pH 7.4 buffer, 7 M urea was adjusted to pH 3.0–3.5 with concentrated HCl and the solution was kept at 4° for a minimum of 3 hr; then solid Tris (6 g/liter) was added, and the pH was adjusted to 7.4

In vitro insulin activity was determined with rat epididymal adipocytes according to Moody *et al.* (8) and Gliemann (9) using monocomponent porcine insulin "Novo" (27 units/mg) as the standard.

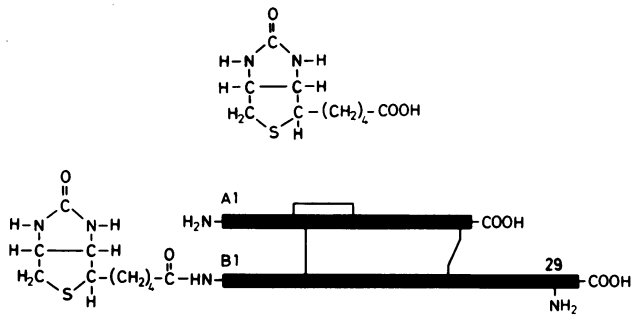

FIG. 1. Structures of biotin (*Upper*) and N^{α,B^1}-biotinylinsulin (*Lower*).

Preparation of N^{α,B^1}-Biotinylinsulin. *N*-Hydroxysuccinimidobiotin (10) (200 mg) was added at room temperature to a stirred solution containing N^{α,A^1}, $N^{\epsilon,B^{29}}$-diBoc-insulin (11) (1 g) and imidazole (200 mg) in dimethyl sulfoxide (30 ml), and stirring was continued for 6 hr. The solution was cooled in an ice bath, and ice-cold water (2 volumes) was added. The resulting solution was desalted on a Sephadex G-25 column (5 × 35 cm) equilibrated with 0.05 M ammonium bicarbonate, and the fractions corresponding to the protein peak were pooled and lyophilized. This material was deprotected with anhydrous trifluoroacetic acid (30 min at room temperature) and the bulk of the trifluoroacetic acid was evaporated at 25°. The residue was dissolved in 7 M urea (50 ml) and the solution (pH 2.5) was applied to a Sephadex G-50 column (5 × 150 cm) equilibrated with 1 M acetic acid. The column was eluted with 1 M acetic acid, and the fractions corresponding to the protein peak were pooled and lyophilized; yield, 800 mg. Desalted protein (1.2 g) was dissolved in 50 ml of 7 M urea/HCl, pH 3.0, and the pH was adjusted to 7.4 (at 4°) by addition of concentrated Tris. The solution was applied to a DE-52 column (5 × 60 cm) equilibrated with pH 7.4 Tris/urea buffer (see General Procedures). The column was developed with a NaCl gradient obtained by mixing 2 liters of pH 7.4 Tris/urea buffer with 2 liters of the same buffer 0.12 M in NaCl. Material corresponding to the major peak was desalted (in three batches) on a Sephadex G-25 column (5 × 35 cm) with 0.05 M ammonium bicarbonate as the eluent. The contents of tubes corresponding to the protein peak were pooled and lyophilized; total yield, 700 mg.

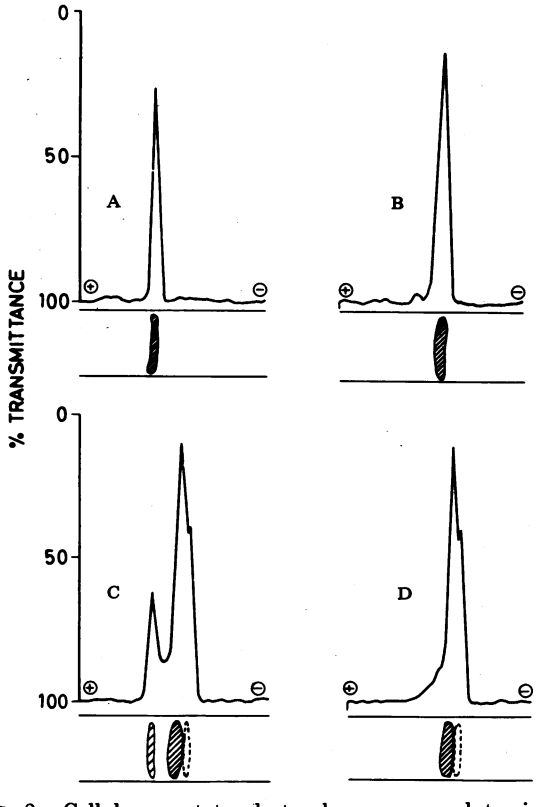

FIG. 2. Cellulose acetate electropherograms and tracings of mixtures of N^{α,B^1}-biotinylinsulin with avidin. (*A*) N^{α,B^1}-biotinylinsulin; (*B*) avidin; (*C*) N^{α,B^1}-biotinylinsulin and avidin (ratio of biotinylinsulin to avidin subunits, 1:1); (*D*) N^{α,B^1}-biotinylinsulin and avidin (ratio of biotinylinsulin to avidin subunits, 1:2). For details, see *Experimental*.

Preparation of Avidin–Sepharose. Activated Sepharose (wet weight, 8.9 g), prepared by reaction of 2,4,6-trifluoro-5-chloropyrimidine with alkali-treated Sepharose (12), was washed repeatedly with 0.05 M sodium bicarbonate buffer at pH 9.0 and slurried in water (final volume, 40 ml). The gel (10 ml of the original slurry; dry weight, 101 mg) was washed repeatedly with 0.05 M sodium bicarbonate in a centrifuge tube, collected on a filter, and placed in a polypropylene tube (1.5 × 10 cm). Avidin (8.5 mg) dissolved in 1.5 ml of 0.05 M sodium bicarbonate, pH 9.0, was added and the tube was slowly rotated for 10 min at room temperature. Sodium chloride was added to a concentration of 0.5 M, and rotation was continued for 12 hr. The resin was collected by filtration, returned to the tube, and rotated for an additional 2 hr in 2 ml of 1 M ethanolamine/HCl pH 9.0. The resin was collected by filtration and the filtrate was checked for avidin by the dye method (6); none was detected. The avidin–Sepharose, in the form of a column, was washed with 20% (vol/vol) aqueous dimethylformamide (50 ml), water (50 ml), and 0.5% NaCl (50 ml). The washed product was collected, suspended in 0.05% (wt/vol) sodium azide, and stored at 4°. Another portion (10 ml) of the original slurry was loaded with 17 mg of avidin in the manner described, and no avidin was detectable in the filtrates.

Determination of Biotin and Biotinylinsulin Binding Capacity. The volume of the sodium azide suspension of avidin-loaded Sepharose was adjusted to 10 ml and 2-ml samples were withdrawn for affinity chromatography. Columns (in pasteur pipettes) were equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and biotin or biotinylinsulin (twice the concentration necessary to saturate the avidin-loaded columns) in 0.05 M ammonium bicarbonate buffer was applied. The columns were eluted with 0.05 M ammonium bicarbonate buffer, pH 9.0, containing 0.5 M NaCl. Fractions (2 ml) were collected and assayed for their biotin content by the dye assay (6). For example, an avidin–Sepharose column (calculated binding capacity, 0.2 μmol of biotinylinsulin, assuming four binding sites per avidin tetramer) was equilibrated with 0.4 μmol of biotinylinsulin and washed in the manner described. Analysis of the effluent and washing showed the presence of 0.203 μmol of unbound biotinylinsulin. Thus, the column contained 0.197 μmol of bound biotinylinsulin. After washing with 250 ml of buffer, this column did not stain with 4-hydroxyazobenzene-2′-carboxylic acid. Biotinylinsulin/avidin–Sepharoses prepared in the same manner were used for the fat cell assays.

RESULTS AND DISCUSSION

The α-amino groups at the termini of the A and B chains and the ε-amino group of lysine B^{29} provide reactive sites for the attachment of insulin to solid supports. Previous methods for the preparation of affinity columns containing insulin have involved addition of unprotected insulin to activated Sepharose

derivatives (13). Such procedures result in the formation of insulin–Sepharoses that are heterogeneous with respect to the bonds connecting the hormone to the support. Advances in the understanding of the chemistry of insulin (14) have opened the way to selective acylation of the molecule, and such methods have now been used to prepare $N^{\alpha,B1}$-biotinylinsulin.

We used $N^{\alpha,A1}$, $N^{\epsilon,B29}$-diBoc-insulin (11) as a convenient starting material for the selective acylation of insulin in position $N^{\alpha,B1}$. Krail *et al.* (15) reacted $N^{\alpha,A1}$, $N^{\epsilon,B29}$-diBoc-insulin with various $N\alpha$-Boc amino acids and, after removal of the protecting groups from the primary reaction products, obtained insulin homologs containing elongated B chains. We have acylated $N^{\alpha,A1}$,$N^{\epsilon,B29}$-diBoc-insulin with *N*-hydroxysuccinimidobiotin (10) and removed the Boc groups from the protected biotinylated intermediate. The ensuing $N^{\alpha,B1}$-biotinylinsulin was purified by ion exchange chromatography. Cellulose acetate electrophoresis at pH 2.2 of biotinyl insulin showed the presence of a major band and a faint minor band. The latter was estimated, by quantitative evaluation of the strips, to represent approximately 1–2% of the product. Electrophoresis of the products of oxidative sulfitolysis of biotinylinsulin produced a normal A chain S-sulfonate and a B chain S-sulfonate that remained at the origin, thus differing significantly from the behavior of native B chain S-sulfonate. Biotinylinsulin binds to avidin specifically, as evidenced by its capacity to displace 4-hydroxyazobenzene-2′-carboxylic acid from the protein. Quantitative determination of the biotin content of biotinylinsulin, using this dye-binding assay (6), gave a value (mean ± SD) of 89 ± 4% of theory. Insulin itself did not interfere with binding since the same value was obtained when the measurements were conducted in the presence of an equimolar quantity of the hormone. Further confirmation of the formation of a complex between the biotinylated hormone and avidin was obtained by cellulose acetate electrophoresis in pyridinium acetate buffer containing urea. The biotinylinsulin–avidin complex migrated at a rate intermediate between that of biotinylinsulin and avidin. Increasing the ratio of avidin to biotinylinsulin brought about a decrease of the biotinylinsulin peak (Fig. 2).

The phenylalanine residue B^1 appears to be located outside the region of the insulin molecule that is intimately concerned with binding to the receptors (for a review, see ref. 16) and acylation of the NH_2-terminal amino group of the B chain causes a much smaller decrease in *in vitro* biological activity than does corresponding acylation of the aminoterminus of the A chain (5, 17). For these reasons, we acylated the insulin molecule with biotin at position $N^{\alpha,B1}$ in order to have available a molecule possessing an unaltered receptor binding region. Based on the experiences just cited, it was not surprising to find that $N^{\alpha,B1}$-biotinylinsulin was as active (mean ± SD, 94 ± 9.6%; *P*, 0.05) as the insulin control in stimulating lipogenesis in rat epididymal adipocytes (8, 9). Neither biotin nor avidin alone exhibited activity in this system. Furthermore, high concentrations of avidin had no effect on lipogenesis when insulin served as the stimulus, but avidin interfered with the biotinylinsulin assay (Table 1). Although the mechanism of this inhibition is not clear, it is significant that at a ratio of biotinylinsulin to avidin of 4:1 (one binding site per biotinylinsulin), at which complex formation is substantial (Fig. 2) even in the presence of urea, 84% of the biological activity was retained. The affinity of biotinylinsulin/avidin–Sepharose for solubilized insulin receptors as well as the effect of free avidin on the formation of biotinylinsulin/insulin–receptor complexes remains to be determined.

Bodanszky and Bodanszky (18) have shown that columns prepared by attaching avidin to cyanogen bromide-activated Sepharose acquire a pink color on exposure to 4-hydroxyazobenzene-2′-carboxylic acid. On titration with biotin, the avidin–Sepharose is bleached and complete decolorization serves as the end point for assessment of avidin loading. We have attached avidin to Sepharose activated with 2,4,6-trifluoro-5-chloropyrimidine and found that the resulting avidin–Sepharose acquires a cherry red color with the dye and that this disappears on addition of biotin or biotinylinsulin.

Table 1. Inhibition of biotinylinsulin-stimulated lipogenesis by avidin

Ratio, biotinylinsulin:avidin	% activity remaining*
1:0	100
1:1	84 ± 5.6
1:5	59 ± 3.5
1:10	39 ± 3.3
1:20	30 ± 4.1

Data shown as mean ± SD.
* P = 0.05. Biotin-binding capacity of avidin sample was 12.61 units/mg.

The accurate determination of the degree of insulin loading of insulin–Sepharose columns poses a problem that is usually solved by determining amino acids present in insulin in acid hydrolysates of the derivatized Sepharose (13). Because of the high carbohydrate content of such hydrolysates, accurate determination of the low concentration of amino acids in them is difficult and the sensitivity of the procedure is rather low. Determination of the loading of avidin–Sepharose with biotinylinsulin is greatly facilitated by the attached biotin which provides a marker whose concentration can be accurately assessed (sensitivity, about 1 nmol) by titration against the avidin–4-hydroxyazobenzene-2′-carboxylic acid complex (6). We estimate the binding capacity of avidin–Sepharose columns by adding an excess of biotinylinsulin and titrating the unbound biotinylated hormone in the column effluents and washings.

Biotinylinsulin attached to avidin–Sepharose beads retains the ability to stimulate rat epididymal adipocytes. The activity of several samples of these beads was about 15% that of free biotinylinsulin, based on the amount of biotinylinsulin anchored to the support. Because the avidin was attached to the Sepharose by a procedure that appears to eliminate the troublesome leakage problems encountered with cyanogen bromide activation (12) and because the noncovalent bonds between biotinylinsulin and avidin are strong, we suspect that the observed activity of the beads is not due to leakage of free biotinylinsulin into the assay medium. Based on repeated beads counts, we have estimated that the assay mixtures contained approximately 10 to 20 beads. These experiments in a heterogeneous system at 37° allow no conclusions to be drawn regarding the interaction of solubilized insulin receptors with biotinylinsulin or biotinylinsulin attached to avidin–Sepharose.

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1. Green, N. M. (1975) *Adv. Protein Chem.* **29,** 85–133.
2. Hofmann, K. & Kiso, Y. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3516–3518.
3. Du, Y. C., Zhang, Y. S., Lu, Z, X. & Tsou, C. L. (1961) *Sci. Sin.* **10,** 84–104.
4. Carpenter, F. H. & Hayes, S. L. (1963) *Biochemistry* **2,** 1272–1277.
5. Brandenburg, D., Gattner, H.-G. & Wollmer, A. (1972) *Z. Physiol. Chem.* **353,** 599–617.
6. Green, N. M. (1970) in *Methods in Enzymology,* eds. McCormick, D. B. & Wright, L. D. (Academic Press, New York), Vol. 18A, pp. 414–424.
7. Bailey, J. L. & Cole, R. D. (1959) *J. Biol. Chem.* **234,** 1733–1739.
8. Moody, A. J., Stan, M. A., Stan, M. & Gliemann, J. (1974) *Horm. Metab. Res.* **6,** 12–16.
9. Gliemann, J. (1965) *Diabetes* **14,** 643–649.
10. Jasiewicz, M. L., Schoenberg, D. R. & Mueller, G. C. (1976) *Exp. Cell Res.* **100,** 213–217.
11. Geiger, R., Schöne, H.-H. & Pfaff, W. (1971) *Z. Physiol. Chem.* **352,** 1487–1490.
12. Gribnau, T. C. J. (1977) *Proceedings of the Symposium "Advances in the Chromatographic Fractionation of Macromolecules,"* Birmingham (England) 1976. The Chemical Society, in press.
13. Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 450–457.
14. Geiger, R. (1976) *Chem. Z.* **100,** 111–129.
15. Krail, G. Brandenburg, D. & Zahn, H. (1975) *Makromol. Chem. Suppl.* **1,** 7–22.
16. Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J. & Gammeltoft, S. (1976) *Nature* **259,** 369–373.
17. Gliemann, J. & Gammeltoft, S. (1974) *Diabetologia* **10,** 105–113.
18. Bodanszky, A. & Bodanszky, M. (1970) *Experientia* **26,** 327.