Photoaffinity Labeling of Insulin Receptor with an Insulin Analogue Selectively Modified at the Amino Terminal of the B Chain†

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ABSTRACT: The direct involvement of the N terminal of the B chain of insulin in the interaction with the insulin receptor on the liver plasma membrane was investigated by using a photosensitive analogue of insulin selectively modified at the N terminal of the B chain. $N^{\alpha A1}$,$N^{\epsilon B29}$-Di(Boc)insulin was reacted for 24 h at 25 °C in the dark with a 16-fold molar excess of the *N*-hydroxysuccinimide ester of *p*-azidobenzoic acid in dimethylformamide and triethylamine to give $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin upon removal of the protecting group with anhydrous trifluoroacetic acid. The derivative was purified on CM-cellulose, and its purity was assessed by polyacrylamide gel electrophoresis. Amino acid analyses, end group determination, and tryptic digests of the purified derivative showed that the N terminal of the B chain was selectively modified. Its photoreactivity was demonstrated by spectral changes and formation of covalent oligomers on exposure to light. Bioassay of the analogue using rat adipocytes showed decreased biological potency (19.83 IU/mg, 75%) compared with that of bovine insulin (26.42 IU/mg, 100%). Receptor binding activity of this derivative to rat liver plasma membrane was also reduced to 53% ($K_d = 6 \times 10^{-8}$ M). The decrease in biological activity is attributable to the decrease in binding affinity to plasma membrane receptor as a complete dose–response curve showed that the derivative achieved the same maximal intrinsic activity as bovine insulin. Immunoreactivity of the analogue was decreased to 85% that of bovine insulin as determined by radioimmunoassay. Radioactive $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin was prepared by iodination with [^{125}I]iodine and chloramine-T. The purity of the radioactive tracer was assessed by polyacrylamide gel electrophoresis using three different systems. Oxidative sulfitolysis of the tracer showed 8–9% of the radioactivity was associated with the B chain. Its photoreactivity and immunoreactivity were established by its covalent specific cross-linking to the heavy and light chains of antiinsulin (bovine) immunoglobulin. The receptor binding activity of the tracer was determined and found to reach maximal binding in 30 min at 24 °C. Rat and mouse (obese and normal) liver plasma membrane preparations were incubated with the $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]-insulin for 30 min at 24 °C in the dark and were subsequently photolyzed. Autoradiography of the $NaDodSO_4$ gel electrophoresis of the solubilized and reduced membranes showed that a radioactive protein of apparent molecular weight 130 000 was specifically labeled in both species of animals.

The formation of a hormone receptor complex on the target cell surface is generally believed to be the first event in a series of molecular interactions that lead to the expressions of the biological effects of a polypeptide hormone, though the details of how the hormone regulates the target cell responses remain incomplete (Catt et al., 1979). Extensive studies have been made on insulin and its receptor interactions (Cuatrecasas & Hollenberg, 1976; Ginsberg, 1977) as well as the isolation and characterization of the insulin receptor (Ginsberg et al., 1976; Jacobs et al., 1977). Structure and function relationship studies of insulin and its analogues suggest that the C-terminal region of the B chain which is involved in dimer formations may also be involved in direct interaction with its receptor on the cell surface (Pullen et al., 1976; Blundell & Wood, 1975). This is substantiated by recent studies on the identification of the insulin receptor in situ by the photoaffinity labeling technique with a photosensitive insulin analogue modified at the ϵ-amino group of the B29-lysine (Yip et al., 1978, 1979, 1980).

More recent studies on the functional role of the N terminal of the B chain of insulin showed that addition of amino acids onto the N terminal of the B chain resulted in a decrease in biological activities (Krail et al., 1975; Yeung et al., 1979b). These studies suggest that the N terminal of the B chain may interact either directly with the insulin receptor or indirectly by affecting other regions of the insulin molecule whose structural and conformational integrity is critical in the receptor interaction. In the present study, we investigate the role of the N terminal of the B chain in interacting with its receptor by photoaffinity labeling techniques. A photosensitive analogue of insulin selectively modified at the N terminal of the B chain with an aryl azide was prepared and characterized. A specific insulin receptor protein on the liver plasma membrane preparation from the rat and obese and normal mouse was identified and labeled with the radioiodinated tracer derived from this photosensitive insulin analogue.

Experimental Section

Materials

Crystalline bovine zinc insulin was a gift from Connaught Laboratories Ltd., Canada. *p*-Azidobenzoyl *N*-hydroxysuccinimide ester (mp 174 °C) was prepared from recrystallized *p*-aminobenzoic acid (mp 191–192 °C) (Sigma Chemical Co.) according to Galardy et al. (1974). Urea from Aldrich Chemicals was dissolved in deionized water to make a 7 M solution which was treated with charcoal and filtered before use. SP-Sephadex C-25, Sephadex G-25 (medium), and Sephadex G-50 (fine) were all obtained from Pharmacia, while CM-cellulose was purchased from Whatman. FDNB[1] and $(Et)_3N$ were obtained from Eastman Chemicals. TPCK-treated trypsin was purchased from Worthington Biochemical. Boc-azide and anhydrous F_3AcOH were obtained from Pierce

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[1] Abbreviations used: FDNB, fluoro-2,4-dinitrobenzene; $(Et)_3N$, triethylamine; F_3AcOH, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; $NaDodSO_4$, sodium dodecyl sulfate; DMF, dimethylformamide; TPCK, tolylsulfonylphenylalanine chloromethyl ketone.

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Chemicals; DMF was purchased from Fisher and was redistilled before use. Carrier-free Na^{125}I and D-[2-^{3}H]glucose were purchased from New England Nuclear Corp. Sodium tetrathionate was prepared according to Lin & Inglis (1972). Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. All other solvents and reagents were of analytical grade and were used without further purification. $N^{\alpha A1}$,$N^{\epsilon B29}$-Di(Boc)insulin was prepared according to the procedure described by Geiger et al. (1971) and was purified according to procedures outlined by Krail et al. (1975). Monoiodinated radioactive bovine insulin for receptor binding assay was prepared by iodination with lactoperoxidase (EC 1.11.1.7) and was separated from noniodinated insulin as described by Hamlin & Arquilla (1974), while radioactive bovine insulin used for radioimmunoassay was prepared by iodination with chloramine-T (Hunter & Greenwood, 1962).

Methods

Analytical Methods. Amino acid analyses were carried out according to the procedure of Spackman et al. (1958) on a Beckman automatic amino acid analyzer, Model 120C, equipped with a manual sample injector. A flow rate of 70 mL/h was used for all analyses. Acid hydrolyses were performed in constant-boiling HCl (Pierce Chemicals) in sealed, evacuated tubes for 18 h at 110 °C. Quantitative end group analyses were performed with FDNB using the procedure described by Africa & Carpenter (1970). Quantitative tryptic digestion was carried out using 0.3 mg of the sample dissolved in 0.2 mL of 0.001 N HCl. An aliquot (0.1 mL) was used for concentration determination on the amino acid analyzer after acid hydrolyses. The remaining portion (0.1 mL) was lyophilized and redissolved in 0.1 mL of 0.05 M Tris at pH 8.0 and digested with 10 μL of trypsin (TPCK–trypsin at 1.5 mg/mL in 0.001 N HCl and 0.001 N $CaCl_2$) at 24 °C for 4 h. Digestion was terminated by adding 0.1 mL of 0.2 M sodium citrate with 2% (v/v) thiodiglycol (25% w/w in water) at pH 2.2. Release of alanine was quantitated by amino acid analysis using 250 mL of the digest. The in vitro biological activity of the insulin derivatives was determined by using isolated adipocytes according to the procedure described by Moody et al. (1974). Receptor binding assay was carried out as described by Yip & Moule (1976) using rat liver plasma membrane. Radioimmunoassay was performed by the back-titration technique described by Wright et al. (1971). The separation of antiinsulin antibody bound with radioactive insulin from the unbound radioactive insulin was achieved by gel filtration on Sephadex G-50 (Yip & Schimmer, 1973).

$N^{\alpha B1}$-(p-Azidobenzoyl)insulin. Purified $N^{\alpha A1}$,$N^{\epsilon B29}$-di-(Boc)insulin (120 mg, 0.02 mmol) was reacted with 82 mg (0.32 mmol) of *p*-azidobenzoyl *N*-hydroxysuccinimide ester for 24 h in 5.0 mL of DMF in the presence of 50 μL of $(Et)_3N$ at 24 °C. The protein was isolated according to the procedure described by Levy & Carpenter (1967). The dried precipitate was subjected to gel filtration on Sephadex G-50 (2.5 × 196 cm) eluted with 0.05 M NH_4HCO_3, pH 8.48. The monomer fraction was pooled, recovered by lyophilization, and treated with anhydrous F_3AcOH (24 °C; 1 h) to remove the protecting groups. The resulting protein was recovered by lyophilization after desalting on a column (2.5 × 30 cm) of Sephadex G-25 in 10% acetic acid, pH 2.22. The crude product was purified by CM-cellulose ion-exchange chromatography in 0.9 M acetic acid containing 7 M urea and 0.015 M NaCl, pH 3.45. The desired fractions were pooled and desalted on Sephadex G-25 (5.0 × 45 cm) in 10% acetic acid, pH 2.22. The product was recovered by lyophilization and was stored in the dark at −20 °C. All the experimental operations were carried out in the dark. Eluant from the column was collected on an LKB UltroRac fraction collector with the timer set at appropriate values, and the fractions were measured at 280 nm. The purity of the derivative was assessed by polyacrylamide gel electrophoresis as described by Poole et al. (1974), modified to obtain 15% gels containing 8 M urea and 0.9 M acetic acid at pH 3.0. The gel was stained with amido black (Racusen, 1973) and was destained with 10% acetic acid.

Photoreactivity of $N^{\alpha B1}$-(p-Azidobenzoyl)insulin. (*a*) *Spectral Change.* One milligram of purified $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin was dissolved in 1.0 mL of 0.05 M NH_4HCO_3, pH 8.48. An aliquot (300 μL) of this solution was added to 1.0 mL of 0.05 M NH_4HCO_3 in a quartz cuvette and was exposed to a sunlamp (250 W; General Electric) at a distance of ~15 cm. At various times (up to 15 min) of exposure to the light source, a spectrum was taken from 420 to 240 nm in a Cary-15 recording spectrophotometer. A control spectrum was taken by using another aliquot (300 μL) of the original solution before and after the sample had remained in the dark for 15 min.

(*b*) *Polymer Formation.* Two milligrams of purified $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin was dissolved in 0.5 mL of 0.05 M NH_4HCO_3, pH 8.48, in a polypropylene microtest tube (Bio-Rad Laboratories). A control dark sample was prepared in a similar manner by covering the tube with aluminum foil. Both the samples and the control tubes were photolyzed at 4 °C in a circulating cold water Lucite bath by using two sunlamps at a distance of ~15 cm. At various times during exposure to the light source, a 50-μL aliquot was removed from the sample and the control and added to 0.5 mL of 0.05 M NH_4HCO_3. These samples were lyophilized and were analyzed on an $NaDodSO_4$ gel system as described by Busse & Carpenter (1976).

Preparation and Characterization of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]insulin. $N^{\alpha B1}$-(*p*-Azidobenzoyl)insulin (0.5 mg) was dissolved in 0.5 mL of 0.001 N HCl. Ten microliters of this solution was reacted with 1 mCi of carrier-free ^{125}I and chloramine-T as described by Hunter & Greenwood (1962). The iodinated mixture was purified on a cellulose powder (Whatman CF-11) column (0.5 × 6 cm) eluted with normal guinea pig serum preincubated with iodoacetamide. The purified radioactive tracer was stored at −20 °C in the dark in 0.1-mL lyophilized aliquots. The purity of the radioactive tracer was examined by three different polyacrylamide gel electrophoretic systems, namely, the $NaDodSO_4$ gel described for insulin by Busse & Carpenter (1976), the acid gel (15%) described above, and gel (15%) at basic pH 9.2 in urea (Zimmerman & Yip, 1974).

Oxidative Sulfitolysis of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]-insulin. Purified $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin was dissolved in 0.4 mL of buffer containing 7 M urea and 0.1 M Tris at pH 7.5, and 25 mg each of Na_2SO_3 and sodium tetrathionate was added. The mixture was stirred at 24 °C in the dark. At the end of 1 h, the reacting mixture was desalted on a Sephadex G-25 column (1.7 × 8.5 cm) eluted with 0.05 M NH_4HCO_3 at pH 8.48. The void volume fractions were pooled and lyophilized. The lyophilized product was then applied to a CM-cellulose column (2.5 × 40 cm) and eluted with 7 M urea in 0.9 M acetic acid at pH 3.45 to determine the degree of iodination of the B chain.

Photoreactivity and Immunoreactivity of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]insulin. Binding of $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin (4.1 μCi) to antiinsulin serum was carried out in 0.15 mL of phosphate-buffered saline (0.2 M phosphate buffer, pH 7.0, containing 0.4% NaCl), using 3 μL of a 1:32

dilution of guinea pig antiinsulin (bovine) serum. The reacting mixture was incubated in the dark at 37 °C for 1 h. Photolysis was carried out as described above. The antibody–antigen complex was precipitated by incubating in the dark with 200 μL of rabbit anti-γ-globulin (guinea pig) serum first at 37 °C for 10 min and then at 4 °C for 16 h. The immunoprecipitates were washed once with 0.5 mL of phosphate-buffered saline and were reduced by boiling for 30 min in 2% $NaDodSO_4$ and 10 mM dithiothreitol. Aliquots of the reduced products were analyzed by disc gel electrophoresis in $NaDodSO_4$ according to procedures of Weber & Osborn (1969) using 11% gel. The tube gel was cut into 1.5-mm sections for the determination of radioactivity in a γ-scintillation counter.

Preparation of Liver Plasma Membrane. Plasma membrane was prepared according to the method of Neville (1968) from the livers of 100-g male Sprague–Dawley rats and 2–3-month-old C57 B1/6 lean and obese mice (ob/ob).

Enzymatic activities of 5′-nucleotidase (Heppel & Hilmoe, 1955) and Na/K-ATPase (Uesugi et al., 1971) were assayed as marker enzymes. The protein concentration of the plasma membrane preparation was determined by the Lowry method as modified and described by Markwell et al. (1978).

Binding of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]insulin to Liver Plasma Membrane. Binding of $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]-insulin to rat liver plasma membrane was studied at 24 °C in the dark in the presence or absence of excess bovine insulin (50 μg) in 0.45 mL of Krebs–Ringer bicarbonate buffer (KRB), pH 7.4. For kinetic studies, ~2 mg/mL membrane protein and 13 μCi of the radioactive tracer were used. Duplicate aliquots of 50 μL were taken at zero time and at 5, 15, and 30 min during incubation and were added to 300 μL of 2.5% BSA in KRB in the small microfuge tubes (Beckman). The mixture was centrifuged in a Beckman microfuge for 5 min. After removal of the supernatant, the pellet was washed with 250 μL of 10% sucrose in KRB. After centrifugation, the supernatant was removed and the radioactivity associated with the pellet was determined in a γ-scintillation counter.

For photoaffinity labeling, the reaction mixture in a polypropylene microfuge tube was photolyzed after 30 min of incubation. The reaction mixture consists of ~2 mg/mL rat liver membrane protein, or 4.5 and 2 mg/mL normal and obese mouse liver, respectively, and 16 μCi of the radioactive tracer.

The discontinuous buffered $NaDodSO_4$–polyacrylamide slab gel electrophoresis method of Ames (1974) was used to analyze the plasma membrane pellet after photolysis. The pellet was solubilized with 100 μL of 100 mM dithiothreitol in 2% $NaDodSO_4$ by boiling for 30 min. The soluble material obtained after centrifugation in a Beckman microfuge for 5 min was analyzed in 10% gel.

When the specificity of the insulin receptor identified on the plasma membrane was tested, a series of natural and semisynthetic insulin analogues of varying biological activities were used to compete with the radioactive tracer in the binding and photolysis experiments. These analogues include bovine proinsulin, guinea pig insulin, $N^{\alpha B1}$-(L-methionyl)insulin, $N^{\alpha B1}$-(L-arginyl)insulin, $N^{\alpha A1}$,$N^{\epsilon B29}$-di(Boc)insulin, $N^{\alpha A1}$,-$N^{\epsilon B29}$-carbonylbis(L-methionyl)insulin, bovine A-chain tetrasulfonates, and bovine B-chain disulfonates.

After staining with either coomassie blue or amido black and destaining, the slab gel was dried for autoradiography on Kodak RP Royal X-OMat film by using a Du Pont Cronex Lighting-Plus intensifying screen (Swanstrom & Shank, 1978).

Results and Discussion

Purification and Characterization of $N^{\alpha B1}$-(p-Azidobenzoyl)insulin. The elution profile for purification of

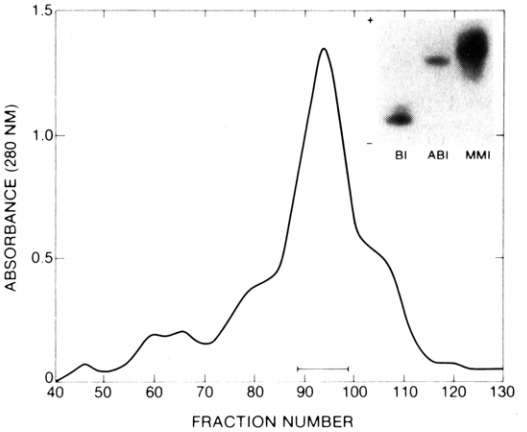

FIGURE 1: Elution profile of $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin purified on a CM-cellulose column (2.5 × 40 cm); buffer, 7 M urea, 0.9 M acetic acid, and 0.015 M NaCl, pH 3.45. Fractions (5.3 mL/fraction) were pooled as indicated. (Insert) Polyacrylamide gel electrophoresis: 15% gel, 8 M urea, and 0.9 M acetic acid, pH 3.0. BI, bovine insulin; ABI, $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin; MMI, $N^{\alpha B1}$-(*N*-mesyl-L-methionyl)insulin.

$N^{\alpha B1}$-(*p*-azidobenzoyl)insulin on CM-cellulose is shown in Figure 1. The fractions were pooled as indicated, desalted, and recovered by lyophilization. The purity of the derivative was assessed by polyacrylamide gel electrophoresis. The electrophoretic system used (15% gel, 8 M urea, and 0.9 M acetic acid, pH 3.0) separates insulin derivatives according to the number of net positive charges. The results are shown in the insert in Figure 1. Both $N^{\alpha B1}$-(*N*-mesyl-L-methionyl)insulin (Yeung, 1976) and $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin had one of the three amino groups protected and moved slower than bovine insulin toward the cathode. As apparent from the gel, the derivative was considered homogeneous.

In bovine insulin, the α-amino groups of glycine and phenylalanine and the ε-amino group of lysine are the three functional amino groups. These amino groups will react with FDNB at pH 8.0. The recovery of glycine, phenylalanine, and lysine from FDNB-treated insulin after acid hydrolysis will be decreased by one residue each when compared with insulin not treated with FDNB. By comparison of the amino acid compositions of the $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin that is treated and not treated with FDNB, the exact location of the amino group where chemical modification has occurred is established. The results as presented in Table I showed that the recoveries of glycine and lysine from the derivative treated with FDNB after acid hydrolysis were decreased by one residue each compared to the untreated one, whereas the recovery of phenylalanine was the same on the FDNB-treated and the untreated one. The result suggested that the α-amino group of Al-glycine and the ε-amino group of B29-lysine in the derivative were free to react with FDNB and were therefore unmodified. On the other hand, the α-amino group of B1-phenylalanine was unavailable for reaction with FDNB and was therefore the site of selective chemical modification. Amino acid analyses of the derivatives not treated with FDNB showed the correct and expected composition. The apparent low recovery of tyrosine and serine may be due to the time-dependent destructions of these amino acid residues by acid hydrolysis. Recovery of alanine from trypsin-treated $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin is 97% compared with that from bovine insulin after correction for protein concentration. Within experimental error, the data showed that the ε-amino group of B29-lysine of the derivative remained free and unmodified. The yield of the purified $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin was ~20% and was comparable to the yields of other analogues

Table I: Amino Acid Analyses of $N^{\alpha B1}$-(*p*-Azidobenzoyl)insulin with (+) and without (−) FDNB Treatment

	no. of residues[a]			
	$N^{\alpha B1}$-(*p*-azido-benzoyl)insulin		bovine insulin[b]	
amino acids	−	+	−	+
lysine[c]	0.98		1	
histidine	2.09	0.36	2	
arginine	1.03	0.94	1	1
aspartic acid	3.10	3.12	3	3
threonine	0.96	1.04	1	1
serine	2.47	2.07	3	3
glutamic acid	6.90	6.88	7	7
proline	1.08	1.06	1	1
glycine[c]	3.98	3.02	4	3
alanine	3.07	2.79	3	3
half-cystine	5.04	4.00	6	5
valine	4.25	4.22	5	5
methionine				
isoleucine	0.57	0.57	1	1
leucine	5.80	5.51	6	6
tyrosine	3.33		4	
phenylalanine[c]	3.05	2.80	3	2

[a] Based on aspartic acid + glutamic acid = 10; an average of three analyses, not corrected for time-dependent destruction. [b] Theoretical values, taken from Ryle et al. (1955). [c] Amino acid residues of interest.

prepared by the same procedure (Yeung et al., 1979b).

The biological properties of the purified $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin were examined in the rat fat cell assay, receptor binding to rat liver plasma membrane, and radioimmunoassay. On the basis of the fat cell assay, the derivative has reduced biological activity, 19.83 IU/mg (95% confidence limits; 18.34–21.44 IU/mg; $\lambda = 0.04$) or 75% as potent as the bovine insulin standard (26.42 IU/mg). The complete dose–response curve (Figure 2a) showed that the derivative has retained its maximal intrinsic activities. The decrease in biological potency of this derivative may be due to an apparent decrease in its binding affinity to the insulin receptor. This notion is substantiated by data obtained from the receptor binding assay as shown in Figure 2b which indicates that $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin with a binding affinity of $\sim 6 \times 10^{-8}$ M was less efficient than insulin (or 53% as potent as insulin) in competing for binding to plasma membrane. The magnitude of decrease in binding correlates well with the decrease in biological activity. Immunoreactivity of this derivative was estimated to be 85%.

Photoreactivity of $N^{\alpha B1}$-(p-Azidobenzoyl)insulin. The photoreactivity of the purified $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin was examined by spectral changes and time-dependent formation of polymers of insulin on exposure to light. As shown in Figure 3, over 40% decrease of absorbance at ~276 nm was observed on exposure of the derivative to the light source for only 1 min. The difference in spectra obtained after 8- and 15-min intervals is minimal. In contrast, without exposure to light, essentially no change in the spectral properties was observed (Figure 3, control). The apparent slight red shift in the absorption maximum from 272 to 276 nm may be attributed to a more nonpolar environment of the aromatic residue in $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin as a result of exposure to light. This change in the microenvironment of the residue would be facilitated by a covalent intermolecular cross-link of the insulin monomer. Such a cross-linking was confirmed by results obtained from time-dependent formation of polymers of insulin on exposure to light (Figure 4). Formation of dimers of insulin was evident after 5 min of exposure to light, and trimers were observed after 15 min of exposure. The

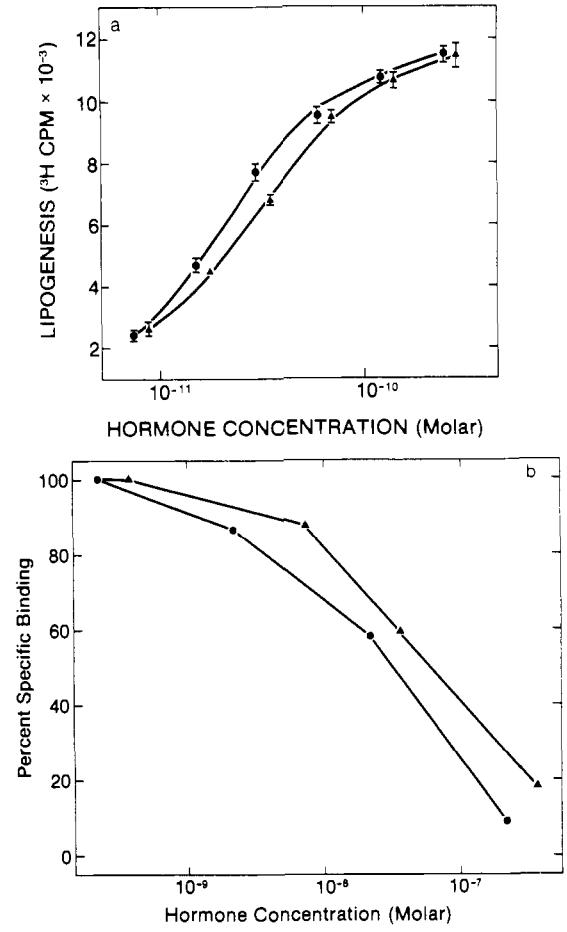

FIGURE 2: (a) Log dose–response curves of $N^{\alpha B1}$-(*p*-azidobenzoyl)-insulin (▲) compared with bovine insulin (●) in the isolated rat fat cell assay. Vertical bars represent standard errors. (b) Competitive receptor binding between bovine ^{125}I-labeled insulin and control bovine insulin (●) or $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin (▲). The amount of specific binding of ^{125}I-labeled insulin in the absence of competing hormone was expressed as 100%. Each point represents the average of duplicate determinations.

formation of such oligomers was light dependent as the control sample (dark) did not show any oligomer formation. The apparent discrepancy between the reactivity of the azido group as measured by spectral changes and that measured by polymer formation is attributable to the fact that a quartz cell was used in the spectral study while a polypropylene microfuge tube was used in the polymerization experiment. Both the spectral changes and the formation of polymers on exposure to light demonstrated the photoreactivity of this analogue and the ability of the azido functional group to survive the preparation and purification procedure, especially the prolonged exposure of the analogue to acidic conditions.

Characterization, Photoreactivity, and Immunoreactivity of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]insulin. The purity of the radioactive tracer was examined by three polyacrylamide gel electrophoretic systems. The basic pH (9.2) system showed that the tracer is essentially homogeneous with small amounts of radioactivity running at the top of the gel (Figure 5a). Figure 5b shows the autoradiograms of the radioactive tracer after polyacrylamide gel electrophoresis at pH 3 (+ to −). The tracer ran slower than iodinated bovine insulin toward the cathode. In addition to the major radioactivity band, a trace amount of radioactivity with slower mobility was detected. $NaDodSO_4$–polyacrylamide gel electrophoresis of the tracer revealed no polymer bands (Figure 5b, autoradiogram; − to +). These data suggest that the radioactive tracer was suf-

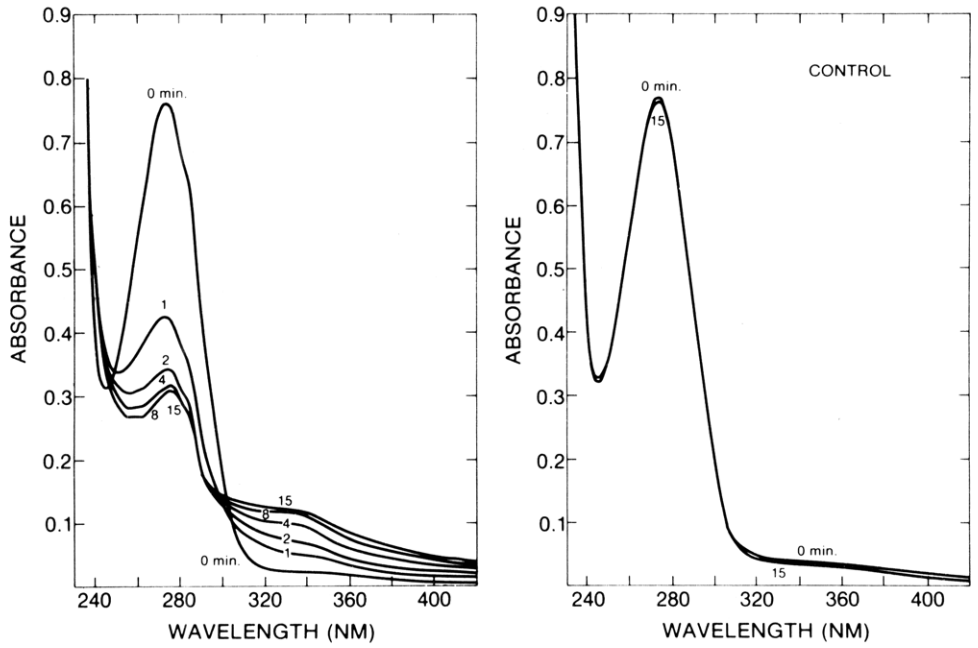

FIGURE 3: Ultraviolet spectral changes of $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin on exposure to light; a time study.

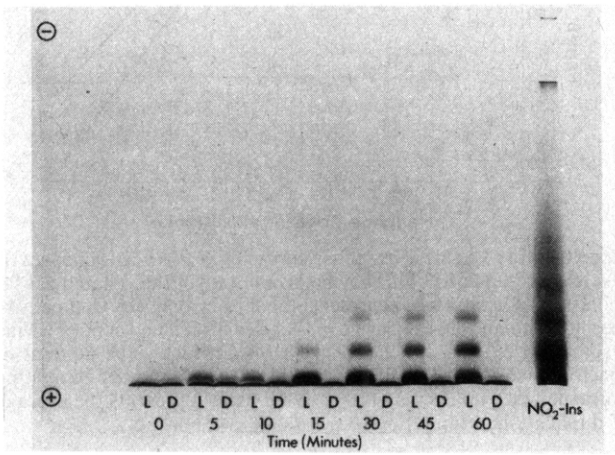

FIGURE 4: Time-dependent polymerization of $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin on exposure to light. NO_2-Ins, nitrated insulin prepared according to Boesel & Carpenter (1970).

ficiently homogeneous in electrophoresis at two different pH values and remained a monomer without any nonspecific cross-linked polymers.

Approximately 90% of the radioactivity in the radioactive tracer was precipitable by the antiinsulin serum. Oxidative sulfitolysis of the radioactive tracer showed that only 8–9% of the ^{125}I label was associated with the B chain.

The photoreactivity of the radioactive tracer was demonstrated by its covalent nonspecific cross-linking to bovine serum albumin (data not shown) as well as its covalent specific cross-linking to the heavy and the light chains of antiinsulin (bovine) immunoglobulin on exposure to light (Figure 6a). As shown in Figure 6a, the photolabeling of the chains of the antiinsulin antibody was inhibited by an excess of native bovine insulin. This is in accordance with the specificity of antibody binding. A dark control (Figure 6b) showed that the labeling process was light dependent. Approximately equal amounts of radioactivity were cross-linked to the heavy and light chains of the antiinsulin antibody. These results demonstrated also that the azido function survived the conditions of iodination and purification.

Binding and Cross-Linking of $N^{\alpha B1}$-(p-Azidobenzoyl)[^{125}I]insulin to Liver Plasma Membrane Proteins. In order to establish that the iodinated tracer retained the ability to bind plasma membrane, a time study of binding was carried out. The radioactive tracer was found to bind rat liver plasma membrane. At the end of 30 min the binding has reached equilibrium and 60% of the total binding was specific; i.e., the binding was abolished by excess bovine insulin.

Binding of the radioactive photosensitive tracer to rat liver plasma membrane, followed by exposure to light, led to covalent labeling of a number of membrane proteins as shown by autoradiography (Figure 7). A major protein band was specifically labeled since the labeling of the band was abolished in the presence of an excess of bovine insulin. Such labeling was the net result of binding to the protein and covalent cross-linking of $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin to the protein through the N terminal of the B chain. The apparent molecular weight of the radioactive band was estimated to be 130 000, bearing in mind the anomalous behavior of glycoproteins in $NaDodSO_4$ gel electrophoresis. The labeling of this membrane protein band was totally dependent on photolysis, since a control not exposed to light showed no such labeling. Other protein bands that were labeled but not displaced by excess bovine insulin probably represented the nonspecific interactions of these membrane proteins with the photoreactive insulin tracer. Bovine [^{125}I]insulin also appeared to label the M_r 130 000 protein band though to a very much smaller extent (Figure 7). This labeling was also light dependent and was abolished in the presence of excess bovine insulin. This observation suggests that minimal light-dependent covalent cross-linking of iodinated insulin to rat liver plasma membrane could occur. As apparent from the autoradiogram shown in Figure 7, considerable radioactivity was located at the top part of the stacking gel, which might represent insoluble nonprotein components of the plasma membrane. Radioactivity at the front of the gel was attributable to the fact that the bulk of the ^{125}I label of $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin was associated with the A chain and that the A chain was cleaved off from the photoreactive insulin derivative after reduction. In addition, $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin bound to the membrane but not covalently linked through the photolabile group at the N terminal of the B chain contributed also to the radioactivity at the gel front.

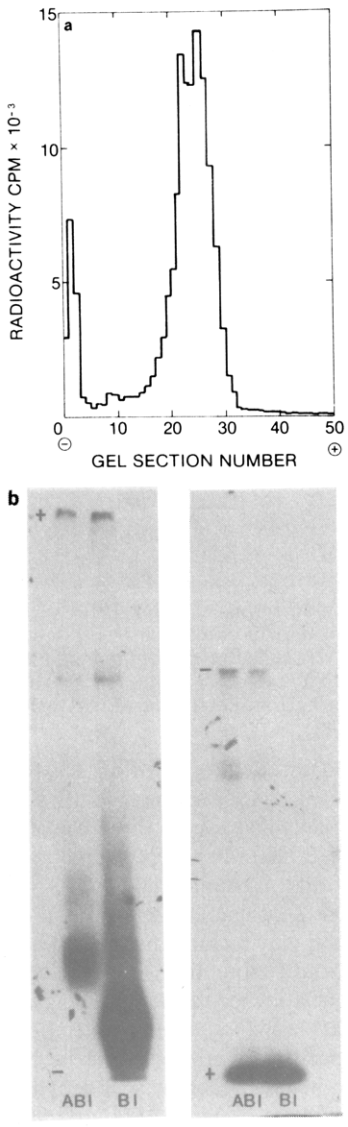

FIGURE 5: (a) Polyacrylamide gel electrophoresis, 15% gel, at pH 9.2, of $N^{\alpha B1}$-(*p*-azidobenzoyl)insulin. The tube gel was cut into 1.5-mm sections, and radioactivity was determined on a γ-scintillation counter. (b) Autoradiograms of polyacrylamide gel electrophoresis at pH 3.0 (+ to −) and NaDodSO$_4$ gel (− to +). BI, bovine [^{125}I]insulin; ABI, $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin.

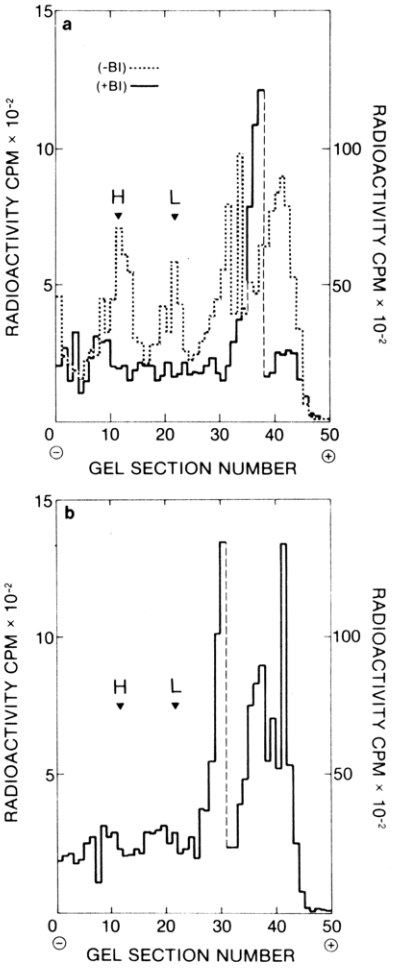

FIGURE 6: Polyacrylamide gel electrophoresis of $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin specifically cross-linked to antiinsulin (bovine) immunoglobulin: H, heavy chain; L, light chain. (a) On exposure to light in the absence and in the presence of excess (50 μg) bovine insulin (BI). From section no. 36 (in the absence of BI) and section no. 39 (in the presence of BI), the ordinate on the right was used. (b) The dark control; from section no. 32, the ordinate on the right was used.

The efficiency of covalent bond formation by aryl azide as photoaffinity labeling is known to be low due to side reactions during photolysis (Knowles, 1972; Baley & Knowles, 1977).

The specificity of the label was further examined by using natural and modified insulin analogues of different biological activities. The biological activities of the derivatives compared to native bovine insulin are as follows: $N^{\alpha B1}$-(L-arginyl)insulin and $N^{\alpha B1}$-(L-methionyl)insulin, 38 and 64%, respectively (Yeung et al., 1979b); bovine A-chain tetrasulfonates and bovine B-chain disulfonates, inactive (Dixon & Wardlaw, 1960); $N^{\alpha A1}$,$N^{\epsilon B29}$-carbonylbis(L-methionyl)insulin, 6.5% (Busse & Carpenter, 1976); $N^{\alpha A1}$,$N^{\epsilon B29}$-di(Boc)insulin, 75% (Geiger et al., 1971); bovine proinsulin, 5% (Gliemann & Sorensen, 1970); guinea pig insulin, 10% (Zimmerman et al., 1974). As shown in Figure 8, the degree of displacement of the label of the protein of M_r 130 000 was in proportion to the biological activities of the analogue used. For example, the extent of labeling of the M_r 130 000 protein in the presence of $N^{\alpha B1}$-(L-methionyl)insulin was less than that in the presence of $N^{\alpha B1}$-(L-arginyl)insulin, hence in agreement with the biological activities of these two derivatives. Similarly, those analogues, natural or modified, whose biological activities are less than 10% of that of bovine insulin have essentially no effect on the displacement of the label of the M_r 130 000 protein. In fact, the intensity of the label was enhanced when binding and photolysis were conducted in the presence of those analogues with low biological activities. The explanation for such an enhancement is not known; however, it might be attributed to the fact that the low biological activity analogues may exert a sparing effect on the radioactive tracer by reducing its loss and nonspecific binding. In general, it appeared that the ability of an insulin analogue to affect the degree of specific labeling of the M_r 130 000 protein was dictated by its biological activity or its binding affinity to the membrane receptor. These observations show that the M_r 130 000 protein labeled by the photoreactive radioactive tracer is a specific insulin receptor.

The photoaffinity labeling of the membrane protein of M_r 130 000 in rat liver plasma membrane by $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin as noted in Figure 7 also applied to obese and lean mouse liver plasma membrane preparations. A membrane protein of similar apparent molecular weight was also specifically labeled. The labeling process is also light dependent (Figure 9). With equal amounts of radioactivity applied to the gel, it appeared that the radioactive band in the lean mouse liver plasma membrane was more intense than that of the obese mouse liver plasma membrane.

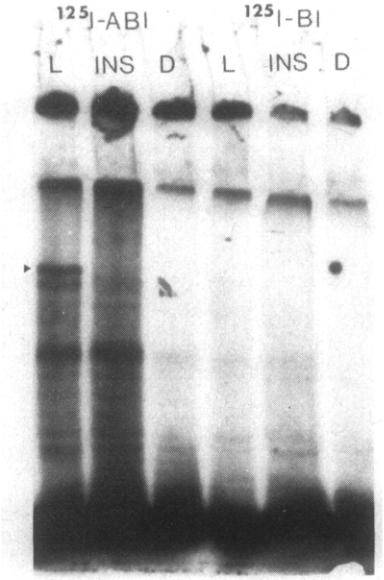

FIGURE 7: Autoradiogram of a $NaDodSO_4$ gel of rat liver plasma membrane labeled with $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin or bovine [^{125}I]insulin. L, exposed to light and in the absence of bovine insulin; INS, exposed to light and in the presence of 50 μg of bovine insulin; D, dark control. ^{125}I-ABI, $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin; ^{125}I-BI, bovine [^{125}I]insulin. The M_r 130000 protein is indicated by an arrowhead.

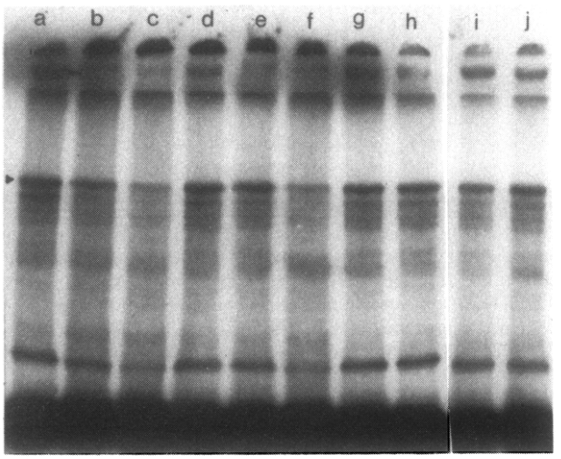

FIGURE 8: Autoradiogram of a $NaDodSO_4$ gel of rat liver plasma membrane labeled with $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin in the presence of 150 ng of natural or modified insulins. (a) No addition, (b) $N^{\alpha B1}$-(L-arginyl)insulin, (c) $N^{\alpha B1}$-(L-methionyl)insulin, (d) $N^{\alpha A1}$,$N^{\epsilon B29}$-carbonylbis(L-methionyl)insulin, (e) $N^{\alpha A1}$,$N^{\epsilon B29}$-di(Boc)-insulin, (f) bovine insulin, (g) bovine A-chain tetrasulfonate, (h) bovine B-chain disulfonates, (i) bovine proinsulin, and (j) guinea pig insulin. This figure is a combination of two different autoradiograms from two separate slab gels run on the same day: slots a–h are from one and slots i–j are from the other. The M_r 130000 protein is indicated by an arrowhead.

Extensive structure and function relationship studies have led to the postulate that residues involved in the dimerization of insulin (i.e., B24–26, B12, and B16) and residues A1-Gly and A21-Asn may be the receptor binding domain for insulin (Pullen et al., 1976). However, recent studies on the chemical modification of the N terminal of the B chain have resulted in insulin derivatives with lowered biological activities and binding affinities to the membrane receptor (Krail et al., 1975; Yeung et al., 1979b). Other studies (Dodson et al., 1979; Insulin Research Group, Academia Sinica, 1974) also suggest that, in addition to the C-terminal region of the B chain, other parts of the insulin molecule may be important in contributing to the full biological activities of insulin. The specific labeling

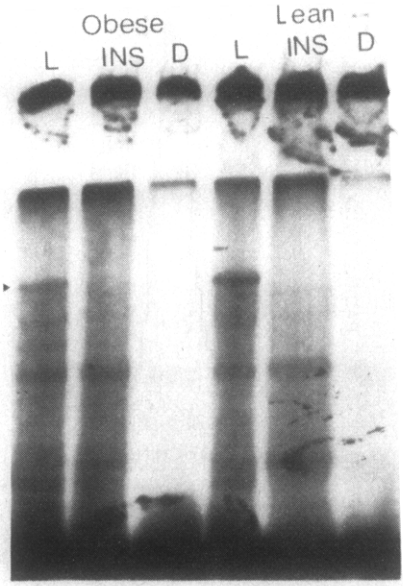

FIGURE 9: Autoradiogram of a $NaDodSO_4$ gel of liver plasma membranes from normal and obese mouse labeled with $N^{\alpha B1}$-(*p*-azidobenzoyl)[^{125}I]insulin. L, exposed to light and in the absence of bovine insulin; INS, exposed to light and in the presence of 50 μg of bovine insulin; D, dark control. The M_r 130000 protein is indicated by an arrowhead.

of a plasma membrane protein of M_r 130000 by the photoreactive insulin analogue selectively modified at the N terminal of the B chain demonstrated a direct involvement of the N terminal of the B chain in receptor binding. Data obtained from photoaffinity labeling and studies of structure and function relationships suggest strongly that the receptor binding domain of insulin may include the N terminal of the B chain.

Our recent study using an insulin analogue with the aryl azide photoprobe attached to the ϵ-amino group of B29-lysine showed that two plasma membrane proteins of M_r 130000 and 90000 were specifically labeled. On the basis of these observations, the insulin receptor was proposed to consist of nonidentical subunits of M_r 130000 and 90000 (Yip et al., 1979, 1980). In the present study, only the M_r 130000 protein was specifically labeled by the analogue with the photoprobe at the B1 N terminal. The labeling of the M_r 130000 protein was also observed with the normal or obese mouse when this derivative was used. Although it remains to be established that the two different photoreactive insulin derivatives labeled the same M_r 130000 protein, it is possible that they interact with the same receptor protein. The specific labeling of only the M_r 130000 protein and not the M_r 90000 protein by the B1 photoreactive insulin may be explained with respect to the spatial arrangements of the α-amino group of the B1-phenylalanine and the ϵ-amino group of the B29-lysine in three-dimensional structure. X-ray crystallographic studies showed that the distance between these two amino groups is probably greater than 20 Å (Blundell et al., 1972). It is possible that the M_r 90000 protein in the insulin receptor complex is topographically located near the ϵ-amino group of B29-lysine but at a distance away from the α-amino group of B1-phenylalanine. The molecular organization of the M_r 130000 and 90000 proteins in the insulin receptor complex could be such that it allows interactions of the ϵ-amino group of B29-lysine to both proteins as a result of the flexibility of the side chain of the lysine molecule (Yip et al., 1980) but only permits interactions of the α-amino group of B1-phenylalanine to the M_r 130000 protein. Currently, photoreactive insulin analogues with the aryl azide attached to different positions of the amino termini are under investigation in our laboratory

in an effort to establish if the M_r 130 000 proteins labeled by the B29 and B1 photoreactive analogues are the same.

Acknowledgments

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