only in the sequence, but also in the folded form. We may then speculate that acidic and basic regions would be available to interact with DNA, and proteins such as histones.

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Synthesis and Properties of Carbonylbis(methionyl)insulin, a Proinsulin Analogue Which Is Convertible to Insulin by Cyanogen Bromide Cleavage[†]

Wolf-Dieter Busse[‡] and Frederick H. Carpenter*

ABSTRACT: The preparation and use of carbonylbis(L-methionine p-nitrophenyl ester) as a reversible cross-linking reagent for insulin are described. The reaction of 1 equiv of reagent with zinc insulin in dimethylformamide in the presence of triethylamine yields as one of the products $N^{\alpha A1},N^{\epsilon B29}$-carbonylbis(methionyl)insulin, (CBM-insulin). The CBM-insulin was characterized by end group analysis and by the products formed on tryptic and chymotryptic cleavage. It possessed 91% of the immunological and 6.5% of the hormonal activity of insulin. Treatment of CBM-insulin with cyanogen bromide (CNBr) in 70% formic acid for 1 h resulted in nearly complete removal of the methionine bridge to yield insulin. A small amount of a side product was removed on DEAE-cellulose at pH 7.2 to give an overall recovery of insulin of 70–80%. Oxidative sulfitolyses of CBM-insulin gave the hexa(S-sulfonate) which was reduced with dithiothreitol to yield reduced CBM-insulin. The latter compound, containing 6 sulfhydryls, exhibited a pH-dependent circular dichroic spectrum. The form at pH 10 exhibited a spectrum typical of random coil which was converted to a form at pH 7.8 which was characterized by a negative extremum at 213 nm. The change in the spectrum at 213 nm with pH was characterized by an apparent pK_a of 8.5. Studies on the reoxidation of reduced CBM-insulin were performed at pH values between 7.8 and 10 and at protein concentrations of 0.01–1 mg/ml. The best yields (ca. 85%) of the correctly paired disulfide bonds were obtained in reoxidations at pH 9.5–10 at protein concentration of 0.01–0.1 mg/ml. CBM-insulin, which had been isolated from reoxidation at high pH of the reduced CBM-insulin, was cleaved by CNBr to yield a fully active insulin in an overall yield of 60% from the reduced CBM-insulin.

The present paper elaborates on preliminary communications (Busse and Carpenter, 1974; Busse et al., 1974; Busse, 1975) on the synthesis and application of a new bifunctional "methionine" reagent, carbonylbis(methionine p-nitrophenyl ester), which may be used for intra- and intermolecular cross-linking of the amino groups of insulin. From the reaction of insulin (**1**) with 1 equiv of $CO(Met-ONp)_2$,[1] an in-

[1] The abbreviations for amino acid derivatives and peptides are those proposed by the IUPAC-IUB Commission on Biochemical Nomenclature (1972): Boc, *tert*-butyloxycarbonyl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; ONp, *p*-nitrophenyl. In addition, the following abbreviations are used: CBM-, carbonylbis(L-methionyl); CBM-insulin, insulin which is cross-linked by the CBM residue between the α-amino group of glycine A1 and the ϵ-amino group of lysine B29; CD, circular dichroism; CO(Met-ONp)$_2$, carbonylbis(L-methionine p-nitrophenyl ester); CNBr, cyanogen bromide.
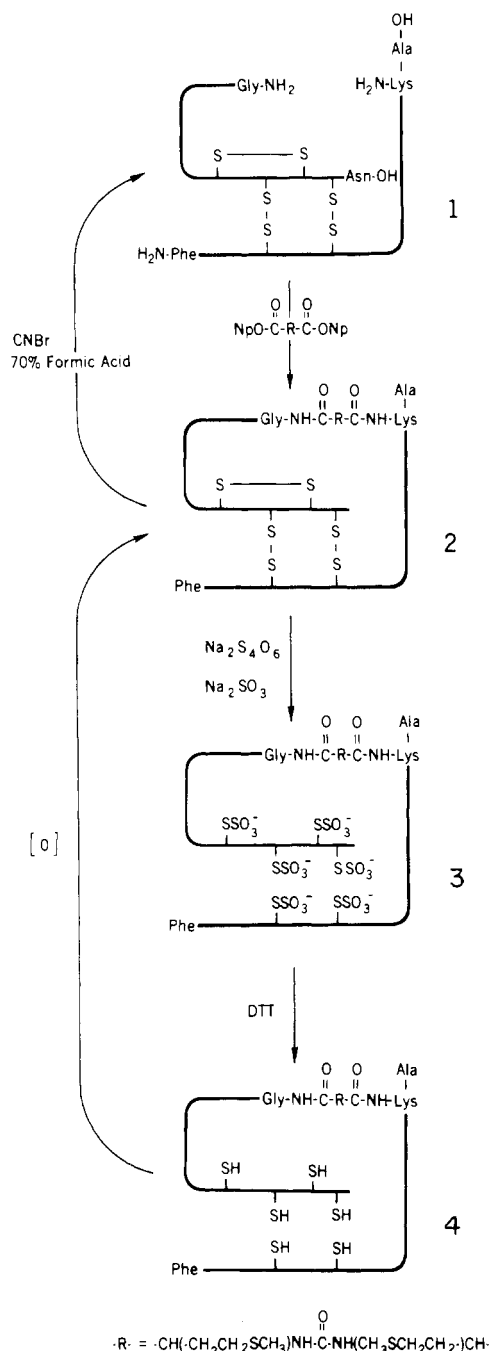
$$-R- = -CH(-CH_2CH_2SCH_3)NH-C-NH(CH_3SCH_2CH_2-)CH-$$

FIGURE 1: Diagram showing the synthesis of CBM-insulin (2) by the reaction of insulin (1) with the cross-linking reagent, carbonyl-bis(methionine p-nitrophenyl ester), followed by oxidative sulfitolysis of the CBM-insulin (2) and reduction of the CBM-insulin hexa(S-sulfonate) (3) with dithiothreitol (DTT) to yield the reduced CBM-insulin (4). The active hormone (1) is regenerated by reoxidation of the reduced CBM-insulin (4) followed by CNBr treatment of the reformed CBM-insulin (2).

tramolecular cross-linked derivative may be isolated in which the CBM residue is attached between the α-amino group of glycine A1 and the ϵ-amino group of lysine B29 and which is designated as CBM-insulin (2). Treatment of the CBM-insulin (2) with CNBr in 70% formic acid (Gross and Witkop, 1961; Gross, 1967) regenerates insulin in 70–80% yield (Figure 1). The synthesis of insulin derivatives cross-linked between the amino groups of glycine A1 and lysine B29 with a reversible cross-linking reagent has potential in the development of a practical synthesis of insulin.

The background for this approach has been described in our earlier communications (Busse and Carpenter, 1974; Busse et al., 1974). In essence it depends on the development of a proinsulin analogue in which correct pairing of the disulfide bonds is facilitated by an intra- rather than an intermolecular reaction and is derived from the work of Zahn and Meienhofer (1958), Lindsay (1972), Brandenburg (1972), Brandenburg et al. (1973a), Robinson et al. (1973), and Brandenburg and Wollmer (1973) on the preparation and properties of intramolecularly cross-linked insulins. These earlier studies used cross-linking residues which could not be removed. This disadvantage has been overcome in the present studies in which it has been demonstrated that the disulfide bonds in CBM-insulin can be reduced and then reoxidized to give, under certain conditions, a 68–85% recovery of the CBM-insulin (with correct pairing of disulfide bonds) which in turn can be converted back to insulin by CNBr cleavage (Figure 1). Another solution to this problem has been presented by Geiger and Obermeier (1973) and Brandenburg et al. (1973b) both of whom used the di(Boc)α,α'-diaminosuberoyl moiety as a cross-linking residue. After reduction–reoxidation of the cross-linked insulin, the Boc groups were removed in anhydrous trifluoroacetic acid and the resulting diaminosuberoyl residue was cleaved by an Edman degradation. Geiger and Obermeier (1973) have also presented procedures by which the separate A and B chain S-sulfonates are specifically cross-linked between glycine A1 and lysine B29 with the suberoyl derivative, followed by reduction, reoxidation, and cleavage to yield the hormone. A different strategy to achieve the correct pairing of the disulfide bridges has recently been presented by Sieber et al. (1974) in pioneering work involving the directed stepwise formation of the disulfide bonds at different stages of the synthesis.

Experimental Section

Materials

Bovine zinc insulin (Brunnengraber, W. Germany, No. 1-8-73) was converted to zinc-free insulin by gel chromatography on Sephadex G-50 in 10% acetic acid (Steiner and Oyer, 1967). Phosgene from Matheson was passed through a sulfuric acid drying trap before use. Urea from Fischer or Aldrich was dissolved in distilled water to make a 7 M solution which was passed over a mixed bed ion exchanger (Bio-Rad, AG-501-X8(D)) to remove ammonium isocyanate (Stark et al., 1960). The gel chromatography resins (Sephadex G-25 (fine), G-50 (fine)) were purchased from Pharmacia; the ion exchange resins (DE-32, CM-32) were Whatman products. α-Chymotrypsin (No. 54C404) and trypsin (No. 33E677) from Worthington were used without further treatment. All other chemicals were of analytical grade and obtained from Aldrich, Fischer, Mallinckrodt, or Sigma.

Methods

Amino acid analyses were performed on a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman et al., 1958). Acid hydrolyses were performed in 6 N HCl, 0.1% phenol in sealed, evacuated tubes for 6 h at 120 °C. Alkaline hydrolyses were performed according to Hugli and Moore (1972) in 4.2 N NaOH, 4% starch at 120 °C for 18 h in polypropylene tubes which were contained in sealed, evacuated glass tubes.

Infrared spectra were determined on a Perkin-Elmer

spectrophotometer utilizing potassium bromide pellets. Ultraviolet absorption measurements were made on a Cary-15 spectrophotometer. Optical rotation and circular dichroism measurements were conducted on a Cary-60 recording spectrophotometer at 27 °C. CD spectra were taken at protein concentrations of 0.2 mg/ml in 10-mm path length cuvettes (208-185 nm), 0.4 mg/ml in 10-mm path length cuvettes (250-200 nm), and 0.4 mg/ml in 20-mm path length cuvettes (350-245 nm) in 0.05 M phosphate buffer at pH 7.8, unless otherwise specified.

Polyacrylamide gel electrophoresis was performed in the presence of 8 M urea-0.9 M acetic acid in a system described by Poole et al. (1974) at pH 4 using a 15% acrylamide gel. The gel was stained with amido black (Racusen, 1973). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Ferro-Luzzi Ames (1974) on a slab gel apparatus at pH 8.8 using a 15% acrylamide gel with the exception that the proteins were not treated with a reducing agent.

Ion-Exchange Chromatography. Chromatographic separations were monitored with Uvicord III Absorbtiometer (LKB) measuring the absorption simultaneously at 254 and 280 nm. Separations on DEAE-cellulose (Whatman DE-32) were run in a buffer containing 7 M urea, 0.01 M Tris, and 0.09 M NaCl at pH 7.2. Chromatographies on CM-cellulose (Whatman CM-32) were carried out using a buffer containing 7 M urea, 0.1 M acetic acid, and 0.075 M NaCl.

End-Group Determination. The free amino groups in the insulin derivatives were qualitatively detected by the dansyl technique (Gray, 1967). The quantitative determination was performed with the fluoro-2,4-dinitrobenzene procedure as described by Africa and Carpenter (1970) or by Edman degradation (Doolittle, 1965) and subsequent amino acid analysis.

Rat Epididymal Fat Pad Assay. This assay was performed according to the method of Renold et al. (1960).

Radioimmunoassay. The double antibody assay was carried out according to Hales and Randle (1963) with an immunoassay kit from Amersham-Searle.

Carbonylbis(L-methionine). L-Methionine (74.5 g, 0.5 mol) was dissolved in 400 ml of 3 N NaOH. The solution was cooled to 0 °C and phosgene (27.5 g, 0.275 mol), dissolved in 125 ml of toluene at −40 °C, was added to the vigorously stirred solution. After 10 min the reaction mixture was acidified to pH 3 with 6 N HCl. The precipitate was dissolved in hot 15% acetic acid; the hot solution was treated with activated charcoal, filtered, and cooled to yield needle-shaped crystals (33 g, 37%): mp 169 °C; $[\alpha]^{27}$D +4.8° (c 1, methanol). The infrared spectrum had bands at 1569 (s), 1623 (s), 1704 (m), 1735 (s), and 3360 (s).

Anal. Calcd for $C_{11}H_{20}N_2O_5S_2$: C, 40.7; H, 6.2; N, 8.6; S, 19.8. Found: C, 40.5; H, 6.1; N, 8.7; S, 19.5.

Carbonylbis(L-methionine p-nitrophenyl ester). Carbonylbis(L-methionine) (6.48 g, 20 mmol) and p-nitrophenol (5.56 g, 40 mmol) were dissolved in 40 ml of dimethylformamide, and N,N'-dicyclohexylcarbodiimide (8.24 g, 40 mmol) was added at −20 °C. The solution was stirred for 18 h at 2-4 °C and 200 ml of ethyl ether were added in the cold to precipitate the dicyclohexylurea which was removed by filtration. The activated ester crystallized upon addition of 100 ml of 2-propanol and was recrystallized from tetrahydrofuran (350 ml) and ethyl ether (700 ml) to yield 9.0 g (80%): mp 185-186 °C; $[\alpha]^{27}$D −41° (c 1, dimethylformamide), infrared absorption bands at 1565 (s), 1630 (s), 1755 (s), 3380 (s), 1740 (m).
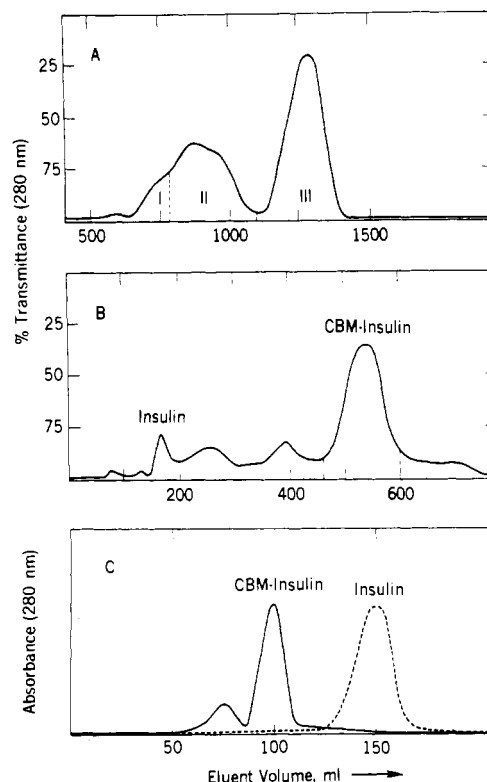

FIGURE 2: Isolation of CBM-insulin (**2**). (A) The crude material obtained from the reaction of insulin with carbonylbis(L-methionyl p-nitrophenyl ester) was applied to a column (5 × 100 cm) of Sephadex G-50 with 10% acetic acid as eluent. The fractions containing mainly intermolecular crosslinked dimers (II) or higher cross-linked oligomers (I) were separated from the monomer fraction III which contained the CBM-insulin. (B) Chromatography of the product obtained from peak III in diagram A on DEAE-cellulose (2.5 × 40 cm) in a buffer containing 0.01 M Tris, 0.09 M NaCl, 7 M urea at pH 7.2. The fractions of the main peak were pooled, dialyzed, and lyophilized. (C) Purification of CBM-insulin (10 mg) by chromatography on CM-cellulose (2 × 30 cm) using a buffer containing 7 M urea, 0.1 M acetic acid, and 0.075 M NaCl at pH 3.9. The main peak represents the CBM-insulin. The dotted line shows the elution pattern of insulin from a control experiment.

Anal. Calcd for $C_{23}H_{26}N_4O_9S_2$: C, 48.8; H, 4.6; N, 9.9; S, 11.3. Found: C, 49.1; H, 4.7; N, 10.1; S, 11.6.

$N^{\alpha A1},N^{\epsilon B29}$-*Carbonylbis(L-methionyl)insulin* (**2**). To a solution of 600 mg of zinc insulin (100 μmol) in 50 ml of dimethyl sulfoxide and triethylamine (150 μl, 1.07 mmol), carbonylbis(L-methionine p-nitrophenyl ester) (56.6 mg, 100 μmol) in 30 ml of dimethyl sulfoxide was added dropwise over 2 h. After the solution had been stirred for 18 h at room temperature, the protein was precipitated by addition of methanol-ethyl ether (9:1), filtered, washed with ethyl ether, and subjected to gel filtration on Sephadex G-50 (5 × 100 cm) in 10% acetic acid (Figure 2A). The fractions were pooled as indicated in Figure 2A, dialyzed against distilled water, and lyophilized (yield: fraction I, 25 mg; fraction II, 115 mg; fraction III, 450 mg). Fraction III was then subjected to ion-exchange chromatography on DEAE-cellulose as shown in Figure 2B. The main peak was dialyzed against distilled water and lyophilized to yield 250 mg (40%) of $N^{\alpha A1},N^{\epsilon B29}$-carbonylbis(L-methionyl)insulin (**2**; CBM-insulin).

CNBr Treatment of Carbonylbis(L-methionyl)insulin. CBM-insulin (12 mg, 2 μmol) was treated with CNBr (120 mg, 1.13 mmol) in 70% formic acid for 1 h at 25 °C. Distilled water (9 ml) was added and the mixture was chroma-
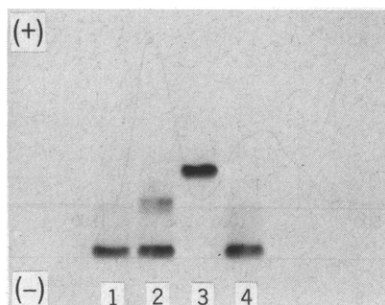
FIGURE 3: Gel electrophoresis at pH 4 of regenerated insulin (1) which was obtained from CBM-insulin after reduction and reoxidation followed by CNBr treatment and purification of the reaction product on DEAE-cellulose, (2) the reaction products obtained after CNBr treatment of CBM-insulin, (3) CBM-insulin, and (4) insulin. The gel was 15% in polyacrylamide; the electrophoresis buffer contained 8 M urea, 0.9 M acetic acid.

FIGURE 4: Separation of the chymotryptic peptides of insulin (A) and CBM-insulin (B) on a column (1.5 × 115 cm) of Sephadex G-25 eluted with 15% acetic acid. The absorption of the eluate was measured with a Uvicord III(LKB) simultaneously at 280 (–) and 254 (- - -) nm. Amino acid analysis of the pooled fractions (see Table I) indicated that the fractions were composed of the following components of: insulin (A) (1) A1-A14 attached to B1-B16, (2) A1-A14 attached to B7-B16, (3) A20-A21 attached to B17-B24(25), (4) B25(26)-B30, and (5) A15-A19; and of CBM-insulin (B) (6) A1-14 attached to both B26-30 and to B1-16, (7) A20-21 attached to B17-25, (8) A15-19, and (9) free tyrosine and phenylalanine. In each case the material in peak (0) was chymotrypsin.

tographed on Sephadex G-25 (2.0 × 40 cm) in 10% acetic acid. The protein fraction was dialyzed against water and lyophilized.

Carbonylbis(L-*methionyl*)*insulin Hexa*(*S-sulfonate*). CBM-insulin was subjected to oxidative sulfitolysis (Bailey and Cole, 1959) and converted to the hexa(S-sulfonate) as described earlier (Busse et al., 1974).

Reduction and Carboxymethylation of CBM-insulin. CBM-insulin (10 mg) or CBM-insulin hexa(S-sulfonate) (10.5 mg) was dissolved in 2 ml of a reduction buffer containing 8 M urea, 0.25% EDTA, and 0.36 M Tris at pH 8.6. Nitrogen was passed through the solution for 30 min and 12 mg of dithiothreitol were added. After 2 h of reaction the pH was adjusted to 8.4 (Crestfield et al., 1963) and 50 mg of recrystallized iodoacetic acid was added. The solution was stirred for another 20 min, dialyzed, and lyophilized to give 8.5 mg of S-carboxymethyl-CBM-insulin. The amino acid analysis of the hydrolysate of the S-carboxymethyl-CBM-insulin showed 5.5 mol of S-carboxymethylcysteine per mole of insulin derivative and no cystine could be detected.

Reoxidation of Reduced CBM-insulin. A solution of reduced CBM-insulin was prepared by desalting of the reduction mixture described above on a column of Sephadex G-25 (2.5 × 40 cm) in 0.05 M phosphate buffer at pH 7.8 (Busse et al., 1974). The solution of reduced CBM-insulin was diluted to the desired volume with 0.05 M phosphate buffer in a 250-ml Erlenmeyer flask which in some cases was coated with bovine serum albumin (Steiner and Clark, 1968). In various experiments the pH was adjusted and maintained at values between 7.8 and 10.0, while the solution was stirred slowly at room temperature. Aliquots were taken at various time intervals for measurement of the CD spectrum and the sulfhydryl content (Ellman, 1959). After complete reoxidation the solution was lyophilized and the products were chromatographed on a column of Sephadex G-50 (2.5 × 150 cm), using 0.05 M ammonium bicarbonate as a solvent.

Tryptic Digestion. To a solution of 4 mg of insulin or insulin derivatives in 5 ml of 0.05 M Tris at pH 8.0, 100 μl of a solution of trypsin (4 mg/ml in 0.001 N HCl, 0.001 M CaCl$_2$) were added and the mixture was incubated for 4 h at room temperature. Aliquots were acidified and subjected to amino acid analysis to determine the release of alanine.

Chymotryptic Digestion. Insulin or CBM-insulin (8 mg) was dissolved in 1 ml of 0.1 M ammonium bicarbonate
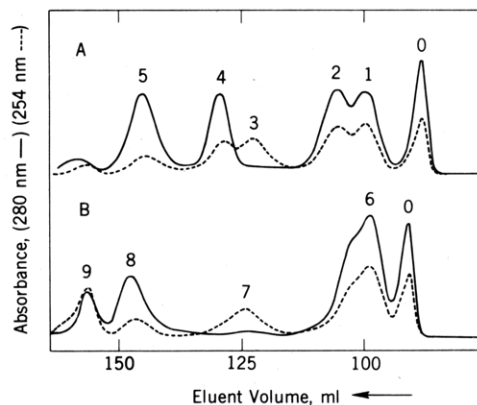
which was adjusted to pH 8.6 with 0.2 N NaOH. Chymotrypsin (1 mg) was added to the solution and the mixture was incubated for 90 min at room temperature. After the addition of 0.1 ml of glacial acetic acid, the solution was subjected to chromatography on a column of Sephadex G-25 (1.5 × 115 cm) which was equilibrated and run with 15% acetic acid.

Results

Preparation and Characterization of Carbonylbis(L-*methionyl*)-*insulin.* The reaction of insulin with carbonylbis(L-methionine p-nitrophenyl ester) was performed under conditions similar to those described by Lindsay (1972) and by Brandenburg (1972) for the reaction of insulin with activated dicarboxylic acids. The isolation of the desired CBM-insulin was performed in three steps through column chromatography: (1) separation according to molecular weight by gel filtration on Sephadex G-50 in 10% acetic acid; (2) separation according to anionic charge on DEAE-cellulose in a buffer containing 7 M urea, 0.1 M Tris at pH 7.2, and 0.09 M NaCl; and (3) separation according to cationic charge on CM-cellulose using a buffer at pH 3.9 containing 7 M urea, 0.1 M acetic acid, and 0.075 M NaCl. The elution patterns of the chromatographies are shown in Figure 2. If a freshly prepared and purified (mixed-bed ion exchange resin treatment) solution of urea was used in the DEAE chromatography, the resulting CBM-insulin was usually free of side products and further chromatography on CM-cellulose was not necessary. However, if commercial urea was used without further purification in the DEAE-chromatography, carbamoylation of amino groups (Stark et al., 1960) occurred to a varying extent. In such cases a further chromatography in acid solution on the CM-cellulose column (Figure 2C) was necessary to prepare products which were homogeneous as determined by polyacrylamide gel electrophoresis (Figure 3, gel 3). The CBM-insulin after steps (1) and (2) was obtained in yields of 35–40% based on the initial insulin.

Amino acid analysis performed on acid hydrolysates of

Table I: Amino Acid Analysis of Insulin and Its Derivatives.

Amino Acid	Insulin[a]	CBM-Insulin[a]	Regner Insulin[a,b]	Products Isolated from the Peaks in Figure 4[c]								
				1	2	3	4	5	6	7	8	9
Asp	3.0	3.0	2.8	1.3		1.0*		1.0	1.2	1.0*	0.9	
Thr	0.9	0.9	0.9				0.9		1.3			
Ser	2.6	2.6	2.4	2.3	2.3				3.2			
Glu	7.0	7.1	7.1	4.0*	3.0*	1.3		2.1	5.1	1.1	1.9	
Pro	1.2	1.2	1.1				0.9		0.6			
Gly	3.9	4.1	3.4	2.1	2.0	1.9			3.2	1.7		
Ala	2.9	3.1	2.8	1.9	1.7		1.0*		4.0			
Cys/2	4.5	4.5	4.5	3.2	2.7	1.0			4.1	1.2		
Val	4.0	4.2	3.8	3.9	2.9	1.1			4.2	1.3		
Met		1.9[d]							1.8[d]			
Ile	0.4	0.5	0.4	0.8	0.8				1.3			
Leu	5.8	5.8	6.5	4.0	3.0	0.9		1.0*	4.1	1.0	1.0*	
Tyr	3.8	3.8	3.5	1.8	1.8		0.8	0.9	3.1		1.0	1.7
Phe	2.7	2.8	2.5	0.8		1.1	0.6		0.8	1.6		1.0*
Lys	1.0	0.9	1.0				1.0		1.5			
His	1.8	1.6	1.9	1.9	1.1				2.0*			
Arg	1.0	0.8	1.0			1.0				0.9		

[a] Values were based on Asp + Glu = 10. [b] Insulin recovered from CNBr treatment of CBM-insulin which had been subjected to reduction and reoxidation and which was purified by chromatography on DEAE-cellulose. [c] The values are the ratios to the amino acid marked with an asterisk (*). [d] Determined in an independent analysis after hydrolysis in 4.2 N NaOH and corrected for losses during hydrolysis.

the model compounds, carbonylbis(methionylglycine ethyl ester) and carbonylbis(methionylphenylalanine methyl ester) (unpublished results), as well as CBM-insulin revealed that a poor yield of methionine was produced during the standard conditions (120 °C for 6 h in 6 N HCl) of acid hydrolysis. This is attributed to the fact that carbonylbis(amino acids) may rearrange under strongly acid conditions to yield hydantoins (Sela and Berger, 1955) which are relatively stable to acid hydrolysis. When the model compounds were subjected to the alkaline hydrolysis conditions similar to those of Hugli and Moore (1972) (18 h at 120 °C in 4.2 N NaOH), approximately 75% of the methionine was released. The CBM-insulin was subjected to amino acid analysis after acid and alkaline hydrolysis with the results shown in Table I. When the values for methionine in the alkaline hydrolysate were corrected for the recovery observed on the model compounds, nearly 2 additional mol of these amino acids were observed in the corresponding cross-linked compounds.

When the CBM-insulin was reacted with excess fluorodinitrobenzene and the product subjected to amino acid analysis (Africa and Carpenter, 1970), 1 mol of phenylalanine was lost but the glycine and lysine values were not affected. This result indicates that the CBM-residue in CBM-insulin is cross-linked between the α-amino group of glycine A1 and the ε-amino group of lysine B29. This was confirmed by the observation that hydrolysates of dansylated CBM-insulin yielded only dansylphenylalanine and no dansylglycine or ε-dansyllysine by the procedure of Gray (1967). No alanine was liberated in tryptic hydrolysis of CBM-insulin under conditions in which insulin yielded 1 mol of alanine. Oxidative sulfitolysis by the procedure of Bailey and Cole (1959) of the CBM-insulin gave only a single polypeptide according to paper electrophoresis at pH 2 using an electrophoresis buffer containing 7 M urea and 2.4 M formic acid.

Chymotryptic cleavage of insulin, CBM-insulin, and insulin regenerated from CBM-insulin was performed according to the procedures described recently (Busse and Gattner, 1973a,b; Levy, 1973). The separation of the chymotryptic peptides by gel filtration on Sephadex G-25 in 15% acetic acid is shown in Figure 4. The amino acid analysis of the isolated products is given in Table I. The results demon-
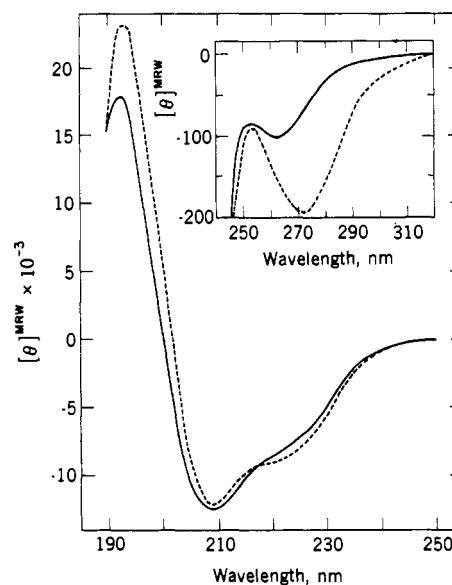

FIGURE 5: CD spectra of CBM-insulin (—) and zinc-free insulin (- - -) in 0.03 M phosphate buffer at pH 7.8.

strate that the C-terminal end of the B chain (B26–B30) is linked to the N-terminal peptide of the A chain in the CBM-insulin. After removal of the carbonylbis(methionyl) bridge from CBM-insulin (see below), the chymotryptic fragmentation of the regenerated insulin was identical with that of the native hormone. The CBM-insulin exhibited a CD spectrum in the low uv which was quite similar to insulin (Figure 5). However, the negative extremum at 275 nm which is present in insulin was much diminished in CBM-insulin.

The CBM-insulin exhibited immunological and hormonal activities which were 91 and 6.5%, respectively, of that of insulin.

CNBr Cleavage of CBM-insulin. CBM-insulin at a concentration of 10 mg/ml in 70% formic acid was treated with a large excess of CNBr for 1–6 h at room temperature. The reaction mixture was desalted on Sephadex G-25 (2 × 25 cm) in 10% acetic acid and the protein peak was lyophilized
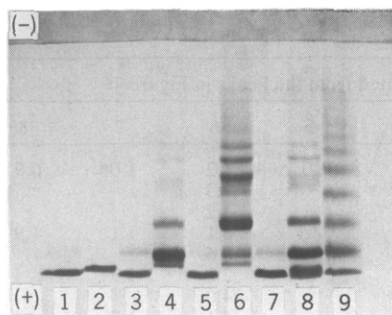
FIGURE 6: Sodium dodecyl sulfate-gel electrophoresis at pH 9.5 in a 15% polyacrylamide gel of (1) insulin, (2) CBM-insulin, (3) CBNr-treated CBM-insulin, (4) dimer fraction (II in Figure 2A), (5) CNBr treated dimer fraction, (6) polymer fraction (fraction I in Figure 2A), (7) CNBr treated polymer fraction, (8) crude reaction mixture of CBM-cross-linked insulin and (9) nitrated insulin as a control. This cross-linked nitrated insulin results from the treatment of insulin with tetranitromethane according to the procedure described by Boesel and Carpenter (1970).
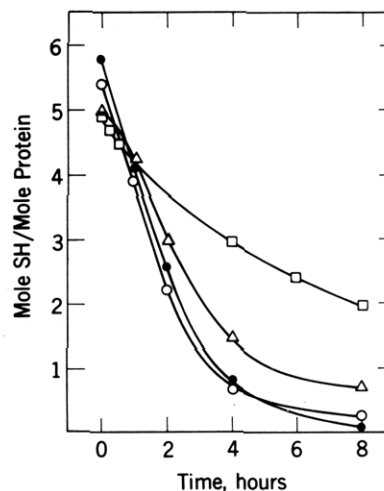

FIGURE 7: Rate of disappearance of sulfhydryls vs. time during the reoxidation of reduced CBM-insulin at 0.1 mg/ml in 0.05 M phosphate buffer at pH 7.8 (□), 8.5 (△), 9.0 (○), and 9.5 (●) at 25 °C.

to give a product from which insulin in form of its zinc complex could be crystallized. As shown by polyacrylamide gel electrophoresis at pH 4, the lyophilized product contains a large amount of insulin and a small amount of a side product which behaves like insulin blocked on one amino group (Figure 3, gel 2). The side product was removed by chromatography on DEAE-cellulose under the same conditions as those used for the isolation of CBM-insulin (Figure 8). The main product which behaved like insulin on the DEAE-cellulose chromatography was isolated in 70–80% yield based on the starting CBM-insulin. Further characterization of this material as insulin was afforded by (1) reaction with fluorodinitrobenzene followed by acid hydrolysis which revealed the loss of 1 mol of phenylalanine, glycine, and lysine; (2) acrylamide gel electrophoresis at pH 4 (Figure 3, gel 1); (3) chymotryptic hydrolysis followed by isolation and characterization of the chymotryptic fragments; (4) immunological assay in which the material responded identically with insulin yielding 25 IU/mg.

As shown in Figure 2A, a substantial portion of the products formed in the reaction of $CO(Met-ONp)_2$ with insulin is composed of substances with higher molecular weights than insulin as judged by their elution pattern on Sephadex G-50. This result indicates that intermolecular cross-linking of the insulin molecules takes place to yield oligomeric products. Such a conclusion is confirmed by the results obtained on sodium dodecyl sulfate-gel electrophoresis as shown in Figure 6. The crude mixture from the reaction of $CO(Met-ONp)_2$ with insulin contains a wide distribution of molecular weights (Figure 6, column 8). Fraction II (Figure 2A) from the Sephadex separation contains dimers and trimers (Figure 6, column 4) and fraction I (Figure 2A) contains trimers and higher oligomers (Figure 6, column 6). Upon treatment of these intermolecular cross-linked derivatives with CNBr, they are converted back to substances exhibiting the same molecular weight as insulin (Figure 6, columns 5 and 7). In the sodium dodecyl sulfate-gel electrophoresis, CBM-insulin runs slightly slower (Figure 6, column 2) than insulin (Figure 6, column 1). After CNBr treatment of CBM-insulin, as well as the intermolecular cross-linked products, the principal component runs like insulin (Figure 6, columns 3, 5, and 7). However, a small amount of material in the CNBr-treated materials appears at the position of dimers. This result indicates that the side reaction, noted above, in the CNBr cleavage of CBM-insu-

lin may involve an intermolecular reaction. It should be noted that, in these sodium dodecyl sulfate-gel experiments, no reducing reagent was used and, therefore, the disulfide bonds are intact. Although the oligomers formed on the treatment of insulin with tetranitromethane (Boesel and Carpenter, 1970) exhibit a straight line relationship between logarithm of molecular weight and R_f from the dimer to n-mer, this does not appear to be the case for the oligomers formed by the cross-linking with $CO(Met-ONp)_2$.

Reduction–Reoxidation Studies with CBM-insulin. To prepare the reduced CBM-insulin, the CBM-insulin hexa(S-sulfonate) (2 mg/ml) was reacted with 20-fold excess of dithiothreitol (Clelands reagent) (Busse et al., 1974). The reduction mixture was desalted on Sephadex G-25 at pH 7.8 which required approximately 45 min. No extensive oxidation of the sulfhydryls was observed during the desalting operation. As shown in Figure 7, the reoxidation at pH 7.8 takes place very slowly. After the reduced protein had been adjusted to the desired final concentration and pH, the rate of reoxidation of the sulfhydryls was determined (Figure 7).

The circular dichroism spectrum of the reduced CBM-insulin at pH 10 is shown in Figure 8. This spectrum is almost identical with that exhibited by the CBM-insulin hexa(S-sulfonate) (Busse et al., 1974; Busse, 1975) and is consistent with a random coil conformation. With decreasing pH an increasingly negative Cotton effect at 213 nm is observed in the reduced CBM-insulin which reaches its maximum negative value at pH 7.8 and remains unchanged at lower pH values (Figure 8). The plot of ellipticity at 213 nm against pH (insert in Figure 8) resembles a typical titration curve with an apparent pK_a of approximately 8.5. At pH values above 8.5 where ionization of the sulfhydryls and phenolic groups begins to occur, the CD spectrum shifts toward that typical of random coils. At values below pH 8.5 where these ionizations are less dominant, a conformation of the reduced CBM-insulin develops which is typified by a CD spectrum with a minimum at 213 nm. CD studies on the effect of pH on the reoxidation of the reduced CBM-insulin (Figure 9) reveal that the best yields of CBM-insulin are obtained when the reoxidations are conducted at high pH where the random coil predominates. During reoxidation of reduced CBM-insulin at pH values of 9.5 or 10, isosbestic points at 204 and 245 nm are observed in the CD
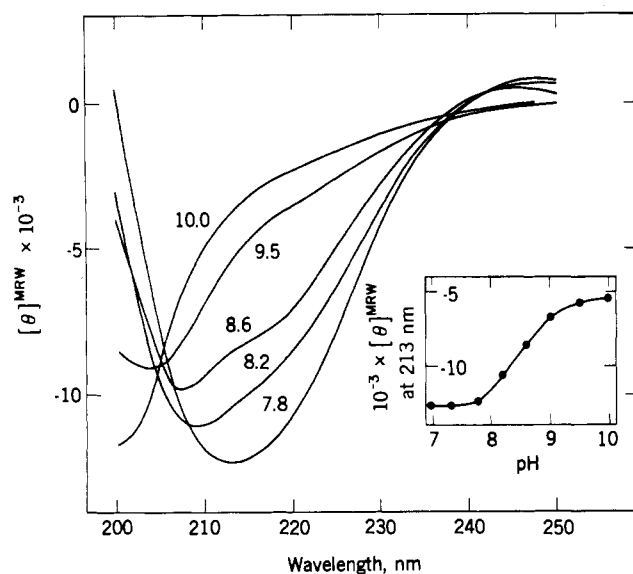
FIGURE 8: CD spectra of reduced CBM-insulin (0.1 mg/ml) in 0.05 M phosphate buffer as a function of the pH indicated by the numbers on the figure. Insert: Mean residue weight ellipticity at 213 nm as function of pH.
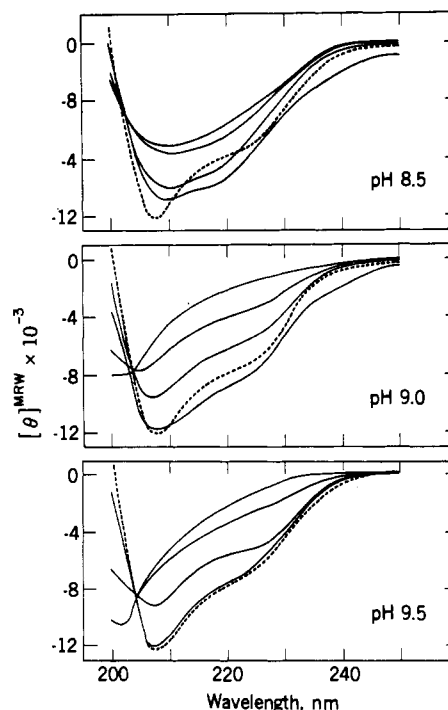

FIGURE 9: Mean residue weight ellipticity as a function of wavelength for the reduced CBM-insulin at a concentration of 0.1 mg/ml in 0.05 M phosphate buffer after various time intervals of reoxidation at pH 8.5, 9.0, and 9.5. The broken lines represent the spectra of the original CBM-insulin. Reading from the top down on each condition the solid lines are the spectra after 0, 1, 2, and 8 h of oxidation time. See Figure 7 for the SH remaining at each time interval.

spectra which indicate that the solution is composed of two species, either reduced CBM-insulin as a random coil or correctly reoxidized CBM-insulin. During reoxidation of reduced CBM-insulin at pH values below pH 9.5, these isosbestic points become more diffuse with decreasing pH and the final reoxidation mixture does not have a spectrum identical with that of CBM-insulin (Figure 9). These results indicate that the conformation assumed by reduced CBM-insulin at pH values below 9.0 and which is typified by the minimum at 213 nm is not the ideal conformation for reforming the correctly paired disulfide bonds.

Characterization of Insulin Regenerated from Reoxidized, Reduced CBM-Insulin. CBM-insulin hexa(S-sulfonate) was reduced as described above and reoxidized at a concentration of 0.01 mg/ml in 0.05 M phosphate buffer at pH 9.5. The lyophilized reoxidation mixture was then subjected to gel chromatography on a column of Sephadex G-50 which was equilibrated and run with 0.05 M ammonium bicarbonate buffer. The main peak was pooled and lyophilized to give 86% of CBM-insulin which was shown to be identical with the starting material according to cellulose acetate and polyacrylamide gel electrophoresis and immunoassay. Treatment of this material with CNBr in 70% formic acid followed by chromatography of the isolated protein on DEAE-cellulose gave 70% of a product (based on the reoxidized CBM-insulin) which was identical with the native hormone in amino acid analysis (Table I), amino end groups, and CD spectrum. The product was identical with insulin in immunoassay (25 IU/mg) and fat pad assay (25 IU/mg). The correct formation of the disulfide bonds was established by chymotryptic digestion followed by gel chromatography of the released peptides on Sephadex G-25 in 15% acetic acid (Figure 4) which gave the same pattern as comparable treatment of native insulin.

Discussion

Procedures in the literature for the synthesis of carbonylbis(amino acids) involve the reaction of amino acid esters with phosgene followed by saponification of the ester group (Fischer, 1902; Wessely and John, 1927; Wessely and

Mayer, 1928; Sela and Berger, 1955) or the reaction of an N-carboxy anhydride of an amino acid with an amino acid ester followed by saponification (Hirschmann et al., 1967). Hydantoins were frequently encountered as side products in these reactions. The principal procedure used in this work involved the direct reaction of amino acids with phosgene in strongly alkaline media to give the desired carbonylbis(amino acids) in modest yield (30–40%).

The reaction of insulin with the bifunctional active ester was performed under conditions similar to those described by Lindsay (1972) and Brandenburg (1972) for the cross-linking of insulin with activated dicarboxylic acids. The observation that such reactions yield substantial quantities of a species which is intramolecularly cross-linked between glycine A1 and lysine B29 has been attributed to the fact that these two amino groups are located quite close to one another (ca. 10 Å) as revealed by the three-dimensional crystal structure of insulin (Adams et al., 1969; Blundell et al., 1971). Systematic studies by Brandenburg et al. (1973a) on the effect of chain length of the cross-linking residue on the CD spectrum have indicated a considerable tolerance of chain length (8 to 16 Å) as judged by this criterion. The CD spectrum of CBM-insulin is quite similar to the spectra of insulins similarly cross-linked with dicarboxylic acids (Brandenburg et al., 1973a; Wollmer et al., 1974). This indicates that the additional side chains present on the CBM residue as compared with the straight chain dicarboxylic acid residues have little effect on the conformation assumed by the cross-linked molecules. The hormonal and immunological properties of the CBM-insulin are also similar to those reported for the derivatives cross-linked with dicarboxylic acids (Gliemann and Gammeltoft, 1974). All exhibit hormonal activities which are about 4–10% of that of in-

sulin while retaining about 90% of the immunological activity. These observations, coupled with the facts that (1) the CD spectra of insulin and the cross-linked insulins, although not identical, are quite similar (Figure 5) and (2) the ability to crystallize the zinc complex of some of the insulin derivatives which are cross-linked with decarboxylic acids (Lindsay, 1972; Brandenburg, 1972; Geiger and Obermeier, 1973; Brandenburg et al., 1973a) suggest (Gliemann and Gammeltoft, 1974) that the cross-linking residues cover a portion of the molecule which is essential for hormonal activity without causing a major change in the tertiary structure. Attempts to crystallize CBM-insulin under a variety of conditions have been unsuccessful. Although little weight can be given to such negative experiments, it may suggest that the methionyl side chain interferes with the normal mode of aggregation of the insulin monomers.

The yields (68–85%) reported here for the recovery of CBM-insulin after reduction followed by reoxidation at pH values of 9.5–10 are somewhat higher than those reported under optimal conditions (pH 8.6) for a similar reaction for proinsulin (Steiner and Clark, 1968). The studies of the effect of pH on the yields in the reoxidation of reduced CBM-insulin demonstrated that, in contrast to similar studies performed on proinsulin, a high pH gave the best yields of correctly paired disulfides. The yields reported here are also better than those reported for reoxidation of a number of cross-linked insulins which were conducted at pH values below 9.5 (Robinson et al., 1973; Brandenburg and Wollmer, 1973; Geiger and Obermeier, 1973; Brandenburg et al., 1973a,b; Wollmer et al., 1974).

The fact that the best yields observed in this work for the formation of the correctly paired disulfide bonds occurred under conditions in which the reduced chain exhibited a CD spectrum consistent with a random coil illustrates a paradox in the rationale for this approach. This rationale was based on the assumption that cross-linking the A and B chains would favor an intra- over an intermolecular reaction and that the reduced A and B chains would possess essentially the same conformation as they exhibit in the cross-linked disulfide form. Holding these two reduced conformers together through a cross-link which defined the correct spatial arrangement of glycine A1 to lysine B29 should favor the correct pairing of the disulfide bonds. The evidence indicates that, at pH values where the sulfhydryls are protonated, the reduced chains assume a conformation which is not the most favorable for the correct pairing of intramolecular disulfide bonds. The best yields are obtained on reoxidation under conditions (pH 9.5–10) in which the chains appear to behave as random coils. Under these reoxidation conditions only two forms, the reduced random coil and the correctly paired reoxidized form, make a significant contribution to the CD spectra. This result suggests that the rate-limiting step in the reoxidation is the formation of the first disulfide bond which acts to constrain the molecule into a conformation in which the other two disulfides are rapidly formed. In any event that part of the rationale which assumed correct folding of the reduced chains appears incorrect, while that part which involved converting an intermolecular to an intramolecular reaction appears sound.

The CBM-insulin which had been subjected to reduction and reoxidation was converted to insulin by treatment with CNBr. The resulting product was indistinguishable from the native hormone in a number of chemical, physical, and biological properties. The fact that fragments obtained on chymotryptic hydrolysis were identical with the native hormone is an unambiguous demonstration that the correct pairing of disulfide bonds occurred during the reoxidation. The best yields observed in this work on the reoxidation of the reduced CBM-insulin (85%) coupled with those obtained in CNBr cleavage of the CBM residue (80%) demonstrate the potential of CBM-insulin as an intermediate in the chemical synthesis of insulin. Current investigations are concerned with developing good procedures for the synthesis of CBM-insulin from separate natural or synthetic A and B chains.

The CBM residue is stable under the conditions of the Edman degradation of insulin (Africa and Carpenter, 1968, 1970) as well as those conditions used for the addition of amino acids to insulin (Levy and Carpenter, 1966, 1967). This has made it possible to perform degradation and synthetic studies on the exposed N-terminal of the B chain. The subject of future communications will be concerned with the preparation of des-Phe[B1]-insulin and its conversion back to insulin and [p-nitrophenyl][B1]-insulin and with other insulin derivatives in which the amino terminus of the B chain has been subjected to stepwise degradation and resynthesis.

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