Possible Involvement of the A^{20}–A^{21} Peptide Bond in the Expression of the Biological Activity of Insulin. 1. [21-Desasparagine,20-cysteinamide-A]insulin and [21-Desasparagine,20-cysteine isopropylamide-A]insulin†

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ABSTRACT: The C-terminal region of the A chain of insulin has been shown to play a significant role in the expression of the biological activity of the hormone. To further delineate the contribution of this segment, we have synthesized [21-desasparagine,20-cysteinamide-A]insulin and [21-desasparagine,20-cysteine isopropylamide-A]insulin, in which the C-terminal amino acid residue of the A chain of insulin, asparagine, has been removed and the resulting free carboxyl group of the A^{20} cysteine residue has been converted to an amide and an isopropylamide, respectively. Both insulin analogues display biological activity, 14–15% for the unsubstituted amide analogue and 20–22% for the isopropylamide analogue, both relative to bovine insulin. In contrast, a [21-desasparagine-A]insulin analogue has been reported to display less than 4% of the activity of the natural hormone [Carpenter, F. (1966) *Am. J. Med. 40*, 750–758]. The implications of these findings are discussed, and we conclude that the A^{20}–A^{21} amide bond plays a significant role in the expression of the biological activity of insulin.

Many insulin analogues have been prepared by modification of the natural hormone or by chemical synthesis. For these analogues, receptor binding affinities and in vitro biological potencies relative to those of natural bovine insulin were the same. These data were interpreted to indicate that the biological activities of the analogues were wholly a consequence of their binding affinity to the insulin receptor; thus, insulin's "binding site" and the region associated with the initiation of cellular processes, the "message region", were indistinguishable (Freychet et al., 1974; Gliemann & Gammeltoft, 1974; Cosmatos et al., 1978). Recent studies, however, from this laboratory, concerning two synthetic insulin analogues as well as the behavior of two naturally occurring insulins, have provided evidence that the region of the insulin molecule responsible for binding to the insulin receptor may be distinguished from that involved in the initiation of the physiological activity of the hormone. Thus, [21-asparaginamide-A]insulin exhibited a biological potency of ca. 13% whereas in receptor binding assays it displayed a potency of ca. 60% relative to insulin (Burke et al., 1980). A similar discrepancy between biological potency, 4–5%, and receptor binding, ca. 25%, has been reported for porcupine (Horuk et al., 1980) and hagfish (Emdin et al., 1980) insulins. In striking contrast, [21-proline-B]insulin (Schwartz et al., 1983) exhibited an inverse behavior, namely, ca. 33% potency in stimulating lipogenesis while in binding assays it showed ca. 17% potency relative to the natural hormone. In this and the following two papers (Chu et al., 1987a,b), we present evidence that implicates a segment of the insulin molecule possibly involved in the "message region" of this hormone. In this paper we present data indicating that the A^{20}–A^{21} amide moiety is a necessary structural element for the expression of biological activity in insulin. We describe the synthesis and biological evaluation of [21-desasparagine,20-cysteinamide-A]insulin ([des-A^{21}Asn,A^{20}-cysteinamide]insulin) and of [21-desasparagine,20-cysteine isopropylamide-A]insulin ([des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin).[1] In both analogues, the C-terminal asparagine residue of the A chain of insulin has been eliminated, and the resulting free carboxyl group of the A^{20} cysteine residue has been converted to an amide and to a monosubstituted isopropylamide, respectively.

Experimental Procedures and Results

Details of materials and analytical procedures used in this investigation are given in a previous publication (Kitagawa et al., 1984). The homogeneity of all the intermediate peptide derivatives was ascertained by thin-layer chromatography on 6060 silica gel (Eastman chromagram sheet) in two solvent systems: chloroform–methanol–water (89:10:1 and 45:10:1). For the enzymatic digestion with leucine aminopeptidase, the method of Hill and Smith (1957) was employed with a chromatographically purified enzyme (Worthington Biochemical Corp.).

^{125}I-Insulin Binding: Liver Plasma Membranes. A fraction enriched in plasma membranes was prepared from the livers of fasted rats essentially as previously described (Horvat et al., 1975). Triplicate incubations, 0.2 mL, contained 40–80 μg of membrane protein, ^{125}I-insulin (Du Pont NEN Products, ca. 100 μCi/μg), varying concentrations of unlabeled bovine insulin or analogue, and sodium phosphate buffer, 0.1 M, pH 7.4, containing 6 mg/mL fraction V bovine serum albumin. After incubation at 24 °C for 45 min, the mixtures were diluted with 2 mL of sodium phosphate buffer, 0.1 M, pH 7.4,

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[1] Abbreviations: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; CM, carboxymethyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide; NP, *p*-nitrophenyl; PMB, *p*-methoxybenzyl; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TEA, triethylamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. Compounds designated by Roman numerals are described fully in the supplementary material (see paragraph at end of paper regarding supplementary material). The IUPAC–IUB name of [21-desasparagine,20-cysteinamide-A]insulin, for example, is des-A21-asparagine-[A20-cysteinamide]insulin (des-Asn^{A21}-[A20-cysteinamide]insulin).

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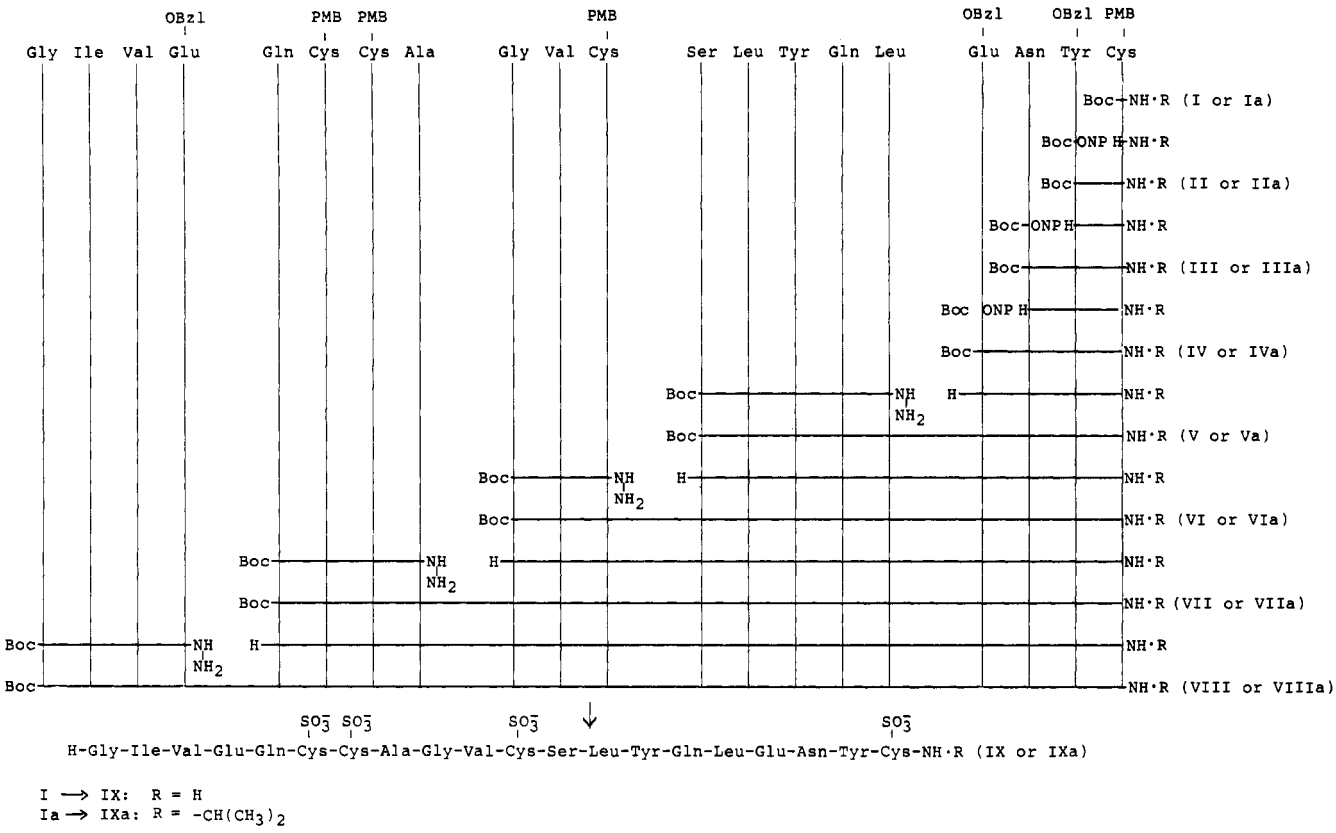
Chart I



I ⟶ IX: R = H
Ia ⟶ IXa: R = $-CH(CH_3)_2$

containing 1 mg/mL fraction V bovine serum albumin, ice cold, and immediately filtered on cellulose–acetate filters (Sartorius 11107). The filters were washed twice with ice-cold buffer, dried, and dissolved in Filtron-X (National Diagnostics, Somerville, NJ) for counting. Nonspecific binding, defined as radioactivity remaining on the filter when incubations contained 1×10^{-5} M unlabeled insulin, amounted to about 10% of the total ^{125}I-insulin bound in the absence of competitor and was substracted from all values. Relative potency was obtained as the concentration ratio of bovine insulin to analogue required to displace 50% of the specifically bound ^{125}I-insulin.

Lipogenesis. Rat adipocytes were obtained by collagenase (1.0 mg/mL, Worthington type II) digestion of epididymal and perirenal fat pads in Krebs–Ringer bicarbonate buffer containing half the recommended calcium concentration, 0.5 mM D-glucose, and 30 mg/mL fatty acid free bovine serum albumin (Boehringer-Mannheim). The gas phase was 95% O_2–5% CO_2. Isolated cells (1.0 mL, 20–40 mg dry weight) were incubated in triplicate in plastic scintillation vials with varying concentrations of bovine insulin or insulin analogue for 45 min at 37 °C before the addition of the label. [3-^{3}H]Glucose (0.5 μCi, Du Pont NEN Products) was added, and the incubation was continued for 1 h. The reaction was stopped by the addition of 5 N H_2SO_4 (0.2 mL), and corn oil (0.2 mL) was added to aid in the extraction of lipids. Soluscint-O scintillation fluid (National Diagnostics, Somerville, NJ) was added to the vials, which were shaken at room temperature for 30 min before counting. Under these conditions <0.01% of the [3-^{3}H]glucose not incorporated into organic-extractable material is counted. Zero and 100% stimulation refer respectively to counts observed in the absence and presence of 9.1×10^{-10} M bovine insulin. Relative potency was obtained as the concentration ratio of natural insulin to analogue required to produce 50% of the maximum stimulation of lipogenesis.

Radioimmunoassay. A commercial kit (Amersham) supplied the reagents for a double-antibody method. Immune precipitates were filtered on cellulose–acetate filters, dried, and counted in Filtron-X. Data were analyzed by the method of Hales and Randle (1963), and relative potency was obtained from the slopes of the resulting straight lines.

General Aspects of the Synthesis of Sheep [Des-A^{21}Asn,A^{20}cysteinamide]insulin and [Des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin. These analogues were prepared by the interaction of S-sulfonated bovine (sheep) B chain with the S-sulfonated forms of [des-A^{21}Asn,A^{20}cysteinamide]A chain (IX) and [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain (IXa) of sheep insulin, respectively, either by the procedures described previously (Katsoyannis et al., 1967b,c) or by the method of Chance et al. (1981). The key intermediate in the synthesis of both A chain analogues was the construction of the protected eicosapeptides (VIII and VIIIa), each containing the entire amino acid sequence of the respective A chain. The synthesis of these eicosapeptides was accomplished by the fragment condensation approach [for a review, see Katsoyannis and Schwartz (1977)]. Removal of the protecting groups from the protected eicosapeptides VIII and VIIIa, was achieved on exposure to 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol (Yajima & Fuji, 1981) according to the modification described recently (Ogawa et al., 1984). The resulting reduced eicosapeptides were converted to the S-sulfonated form (IX and IXa) by oxidative sulfitolysis. The overall synthesis is illustrated in Chart I.

*Glycyl-*L*-isoleucyl-*L*-valyl-*L*-glutamyl-*L*-glutaminyl-S-sulfo-*L*-cysteinyl-S-sulfo-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-sulfo-*L*-cysteinyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-*L*-glutamyl-*L*-asparaginyl-*L*-tyrosyl-S-sulfo-*L*-cysteinamide (Sheep Insulin [Des-A^{21}Asn,A^{20}cysteinamide]A Chain S-Sulfonate) (IX).* The deblocking of the eicosapeptide derivative VIII was carried out on exposure to 1 M TFMSA in TFA solution as described previously (Joshi et al., 1985),

Table I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Leucine Aminopeptidase) of S-Sulfonated [Des-A^{21}Asn,A^{20}cysteinamide]A and [Des-A^{21}Asn,A^{20}cysteine isopropylamide]A Chains

	A^{20} cysteinamide analogue				A^{20} cysteine isopropylamide analogue			
	acid hydrolysis		enzymatic hydrolysis		acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found	theory	found	theory	found
Asp	1	1	0	0	1	1	0	0
Ser	1	0.9	1	*b*	1	0.9	1	*b*
Asn	0	0	1	*b*	0	0	1	*b*
Gln	0	0	2	*b*	0	0	2	*b*
Glu	4	4	2	2	4	4	2	1.9
Gly	2	1.	2	2	2	1.9	2	1.9
Ala	1	0.9	1	0.8	1	1	1	0.8
$^1/_2$-Cys	4	nd[c]	0	0	4	nd[c]	0	0
Val	2	1.7	2	1.9	2	1.5	2	1.7
Ile	1	0.6	1	0.7	1	0.7	1	0.8
Leu	2	2	2	2	2	1.9	2	2
Tyr	2	2	2	2	2	1.9	2	2
S-sulfo-Cys	0	0	4	4.1	0	0	4	3.2[d]

[a] Number of amino acid residues per molecule. [b] Emerge on the same position and not determined. [c] nd, not determined. [d] *S*-Sulfocysteine isopropylamide is not hydrolyzed by leucine aminopeptidase.

and the sulfitolysis of the resulting reduced polypeptide chain was accomplished essentially by the procedure used recently in the synthesis of another A chain analogue (Ogawa et al., 1984). In a typical experiment, a cooled solution, 0 °C of the protected eicosapeptide VIII (230 mg) in 1 M TFMSA in TFA (8 mL) containing thioanisole (1.8 mL) and *m*-cresol (1.3 mL) was stored for 20 min at 0 °C and for 1 h at room temperature. To this solution, cooled to −5 °C, was added dropwise a mixture of 8 M guanidine hydrochloride (28 mL) and concentrated NH_4OH (8 mL) while at the same time the temperature of the reaction mixture was kept below 5 °C. The resulting mixture (pH ~4) was extracted 3 times with ether (50 mL each), and to the aqueous layer, adjusted to pH 8.9 with NH_4OH, were added sodium sulfite (1.6 g) and freshly prepared sodium tetrathionate (0.75 g). After 3.5 h the solution was placed in Spectrapor membrane tubing No. 3 and dialyzed against four changes of distilled water (4 L each) at 4 °C for 24 h. Upon lyophilization of the dialyzate, the crude A chain analogue *S*-sulfonate was obtained as a white powder. This material was dissolved in 0.015 M NH_4HCO_3 (4 mL) and chromatographed on a Sephadex G-15 column (4.5 × 45 cm) equilibrated and eluted with 0.015 M NH_4HCO_3. The effluent corresponding to the main peak, as monitored with an ISCO recording spectrophotometer (Model UA-5), was lyophilized, and the A chain analogue *S*-sulfonate was obtained as a white powder, weight 172 mg. For purification, this material (90 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0, 5 mL) and placed on a Cellex-E column (1.2 × 40 cm) equilibrated with the same buffer. Elution of the column was carried out with a linear NaCl gradient formed by adding to the above buffer (250 mL) 0.6 M NaCl in the same buffer (250 mL). The elution pattern, as monitored with an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 1. The effluent corresponding to the main peak (330–442 mL) was collected, dialyzed as described above, and lyophilized, weight 53.2 mg. Upon rechromatography of this material (101.7 mg) on a Cellex-E column and under identical conditions as described above, the chromatographic pattern shown in Figure 1 was obtained. From the effluent (247–380 mL), after dialysis and lyophilization, the purified sheep insulin [des-A^{21}Asn,A^{20}cysteinamide]A chain *S*-sulfonate was obtained as a fluffy white powder, weight 86.2 mg.

On thin-layer electrophoresis (precoated TLC plates, 10 × 20 cm silica gel 60, EM Laboratories) in 2 N acetic acid–0.6 N formic acid (1:1 v/v), pH 2.0 and 480 V, the synthetic chain

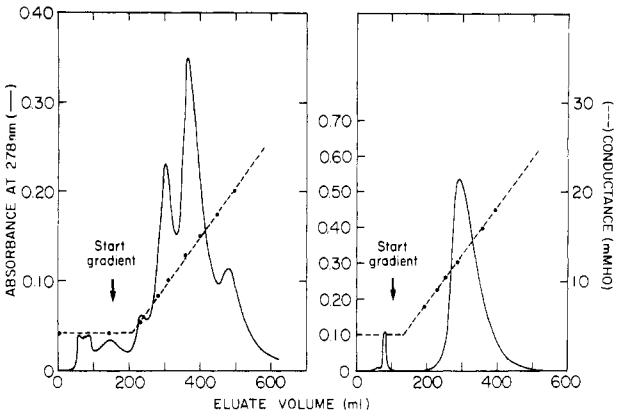


FIGURE 1: (Left) Chromatography of crude [des-A^{21}Asn,A^{20}cysteinamide]A chain *S*-sulfonate on a 1.2 × 40 cm Cellex-E column with 0.1 Tris-HCl buffer (pH 7.0) and a linear NaCl gradient. The column was monitored with an ISCO spectrophotometer and a conductivity meter. (Right) Rechromatography of the product obtained after dialysis and lyophilization of the main peak effluent (330–442 mL) depicted in the left panel.

analogue moved as a single band (data not shown). Amino acid analysis of [des-A^{21}Asn,A^{20}cysteinamide]A chain *S*-sulfonate after acid hydrolysis gave a composition, expressed in molar ratios, in good agreement with the theoretically expected values (Table I). Digestion of the synthetic chain with leucine aminopeptidase and amino acid analysis of the digest gave the molar ratios shown in Table I. It is obvious that the synthetic chain was completely digested by the enzyme, indicating that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes.

Glycyl-L-*isoleucyl*-L-*valyl*-L-*glutamyl*-L-*glutaminyl*-*S*-*sulfo*-L-*cysteinyl*-*S*-*sulfo*-L-*cysteinyl*-L-*alanylglycyl*-L-*valyl*-*S*-*sulfo*-L-*cysteinyl*-L-*seryl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-L-*glutamyl*-L-*asparaginyl*-L-*tyrosyl*-*S*-*sulfo*-L-*cysteine Isopropylamide* (*Sheep Insulin* [*Des-*A^{21}*Asn,*A^{20}*cysteine isopropylamide*]*A Chain S-Sulfonate*) (*IXa*). The deblocking of the protected eicosapeptide VIIIa (200 mg) on exposure to 1 M TFMSA in TFA solution and sulfitolysis of the resulting reduced product were carried out in exactly the same way as described above in the synthesis of compound IX. After Sephadex G-15 chromatography, 147 mg of crude A chain analogue *S*-sulfonate was obtained. For purification, this product (70 mg) was chromatographed on a Cellex-E column (1.2 × 10 cm) with 0.1 M Tris-HCl buffer (pH 7.0) and a

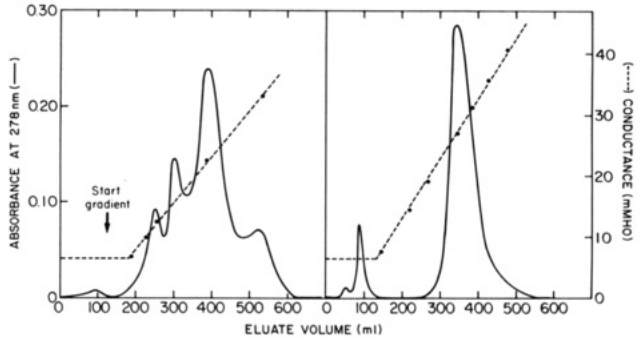


FIGURE 2: (Left) Chromatography of crude [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain *S*-sulfonate. (Right) Rechromatography of the product obtained after dialysis and lyophilization of the main peak effluent (335–470 mL) depicted in the left panel. Chromatography conditions were the same as in Figure 1.

linear NaCl gradient as described in the synthesis of compound IX (Figure 2). The eluate under the main peak (335–470 mL) was dialyzed and lyophilized to give 39.5 mg of A chain analogue *S*-sulfonate. This material (72 mg) was rechromatographed on the same column and under identical conditions as described above (Figure 2). From the effluent (300–470 mL) after dialysis and lyophilization, [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain *S*-sulfonate was obtained in highly purified form, weight 55 mg.

On thin-layer electrophoresis, as above, the synthetic chain moved as a single band. Amino acid analysis after acid hydrolysis and leucine aminopeptidase digestion gave the molar ratios shown in Table I, in good agreement with the theoretically expected values.

S-Sulfonated Derivative of the B Chain of Sheep Insulin. The B chain of sheep insulin is identical with the corresponding chain of bovine insulin (Sanger & Tuppy, 1951; Brown et al., 1955). The bovine (sheep) B chain *S*-sulfonate was prepared by oxidative sulfitolysis of bovine insulin followed by separation of the resulting A and B chain *S*-sulfonates by CM-cellulose chromatography as we have reported previously (Katsoyannis et al., 1967a).

Synthesis and Isolation of Sheep [Des-A^{21}Asn,A^{20}cysteinamide]insulin. This analogue was prepared by the interaction of the S-sulfonated bovine (sheep) B chain with the S-sulfonated sheep [Des-A^{21}Asn,A^{20}cysteinamide]A chain (IX) either by the procedure described previously (Katsoyannis et al., 1967b,c) or by the method of Chance et al. (1981). A typical experiment according to the second route is as follows. A solution of sheep B chain *S*-sulfonate (22 mg), [des-A^{21}Asn,A^{20}cysteinamide]A chain *S*-sulfonate (47 mg), and dithiothreitol (7.6 mg) in 0.1 M glycine buffer (pH 10.6, 10 mL) was stirred at 4 °C for 24 h and then processed as described previously (Katsoyannis et al., 1967b,c). Isolation and purification of the insulin analogue from the combination mixture were carried out by chromatography on a 0.9 × 24 cm CM-cellulose column with an acetate buffer (Na^+, 0.024 M, pH 3.3) and an exponential NaCl gradient according to the procedure we have described previously (Katsoyannis et al., 1967b,c). The elution pattern, as monitored by an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 3. The eluate containing the insulin analogue (216–310 mL) was processed as described previously (Katsoyannis et al., 1967b,c), and the purified [des-A^{21}Asn,A^{20}cysteinamide]insulin was isolated via picrate as the hydrochloride (2.2 mg).

Amino acid analysis of this analogue after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table II). On isoelectric focusing on thin-layer plates (Sephadex IEF) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, [des-A^{21}Asn,A^{20}cysteinamide]insulin focused in one band (Figure 3).

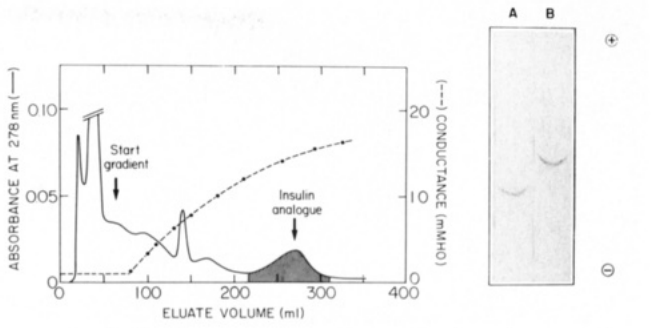


FIGURE 3: (Left) Chromatography of a combination mixture of the [des-A^{21}Asn,A^{20}cysteinamide]A chain *S*-sulfonate with the S-sulfonated bovine (sheep) B chain on a 0.9 × 24 cm CM-cellulose column with acetate buffer (Na^+, 0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored with an ISCO spectrophotometer and a conductivity meter. The insulin analogue (216–310 mL of effluent) was recovered as the hydrochloride. (Right) Paper print of thin-layer isoelectric focusing of synthetic [des-A^{21}Asn,A^{20}cysteinamide]insulin (A) and natural bovine insulin (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing: constant power; 8 W for 4 h.

Table II: Amino Acid Composition[a] of an Acid Hydrolysate of Sheep [Des-A^{21}Asn,A^{20}cysteinamide]insulin and [Des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin

	A^{20} cysteinamide analogue		A^{20} cysteine isopropylamide analogue	
amino acid	theory	found	theory	found
Lys	1	0.9	1	0.9
His	2	2	2	2
Arg	1	1.1	1	1
Asp	2	2	2	2.2
Thr	1	0.8	1	0.9
Ser	2	1.9	2	2.2
Pro	1	1.0	1	1.3
Glu	7	7	7	7.2
Gly	5	4.9	5	5.0
Ala	3	2.9	3	3.2
$^1/_2$-Cys	6	nd[b]	6	nd[b]
Val	5	4.4	5	4.5
Ile	1	0.4	1	0.6
Leu	6	6.3	6	6.2
Tyr	4	4.1	4	4
Phe	3	2.8	3	3

[a] Number of amino acid residues per molecule. [b] nd, not determined.

Synthesis and Isolation of Sheep [Des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin. The synthesis of this analogue by the interaction of the S-sulfonated derivatives of bovine (sheep) B chain and [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain and its purification were accomplished by exactly the same procedure as described above in the synthesis of sheep [des-A^{21}Asn,A^{20}cysteinamide]insulin. Chromatography of a combination mixture of 10 mg of S-sulfonated bovine B chain and 20 mg of [Des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain gave the pattern shown in Figure 4. The insulin analogue was isolated from the effluent (225–310 mL) via picrate as the hydrochloride (1.63 mg).

Amino acid analysis of an acid hydrolysate of this analogue gave the molar ratios shown in Table II, in good agreement with the theoretically expected values. On isoelectric focusing on thin-layer plates (Sephadex IEF) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin focused in one band (Figure 4).

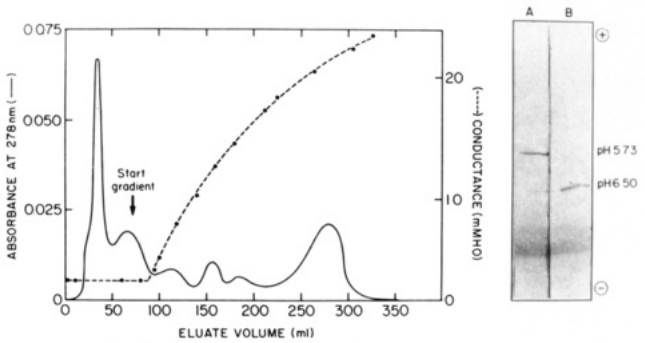


FIGURE 4: (Left) Chromatography of a combination mixture of the [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain *S*-sulfonate with the S-sulfonated bovine (sheep) B chain. (Right) Paper print of thin-layer isoelectric focusing of natural bovine insulin (A) and synthetic [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin (B). Chromatography and isoelectric focusing conditions were the same as in Figure 3.

Biological Evaluation of the Synthetic Insulin Analogues. [Des-A^{21}Asn,A^{20}cysteinamide]insulin displayed a calculated potency of 13.9 ± 3.0% in receptor binding assays and 15.1 ± 2.1% in lipogenesis; for [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin, the potency is 20.2 ± 5.9% in receptor binding and 22.3 ± 2.1% in lipogenesis. These results represent the mean potency ± 1 SD obtained from at least three dose–response curves (not shown) for each analogue. In radioimmunoassay (data not shown), [des-A^{21}Asn,A^{20}cysteinamide]insulin assayed at 4.1% and [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin assayed at 7.7%. All potency values are expressed as percentages of bovine insulin.

Discussion

Previous studies have shown that enzymatic elimination of the A^{21} Asn and B^{30} Ala residues from the insulin molecule resulted in an analogue, [des-A^{21}Asn,des-B^{30}Ala]insulin, displaying less than 4% of the activity of the natural hormone (Carpenter, 1966). A consideration of the procedure used for preparation and purification of this analogue (i.e., carboxypeptidase A digestion of insulin followed by countercurrent distribution) does not exclude the possibility that the insignificant biological activity it displays may be ascribed to contamination with unreacted insulin or other active insulin derivatives. In this connection it is of interest to note that the same preparative procedure but a less rigorous purification of the product (i.e., Sephadex G-75 chromatography) resulted in a product displaying ca. 10% biological activity relative to the natural hormone (Yip & Moule, 1976). It may then well be that the [des-A^{21}Asn,des-B^{30}Ala]insulin is essentially inactive. We have attempted to synthesize [des-A^{21}Asn]insulin by combining synthetically prepared [des-Asn^{21}]A chain with natural bovine B chain in order to establish its potency unequivocally. However, we were unable to effect this combination by all the existing procedures (unpublished data from this laboratory).

The synthetic analogue [des-tripeptide-B^{28-30}]insulin, which lacks the C-terminal tripeptide of the B chain moiety, is equipotent with natural insulin (Katsoyannis et al., 1971). Obviously then, the loss of biological activity of [des-A^{21}Asn,des-B^{30}Ala]insulin may be ascribed solely to the elimination of the A^{21} Asn residue and the concomitant generation of a free carboxyl group at the A^{20} position (Cys).

In view of these considerations it was of considerable interest to synthesize insulin analogues lacking the A^{21} Asn residue but retaining the A^{20}–A^{21} amide moiety. This was accomplished by the synthesis of [des-A^{21}Asn,A^{20}cysteinamide]A chain and [des-A^{21}Asn,A^{20}cysteine isopropylamide]A chain and their ready combination with natural bovine B chain to yield [des-A^{21}Asn,A^{20}cysteinamide]insulin and [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin, respectively. Both these analogues displayed significant receptor binding and equivalent biological activity: [des-A^{21}Asn,A^{20}cysteinamide]insulin ca. 14% and [des-A^{21}Asn,A^{20}cysteine isopropylamide]insulin ca. 20% relative to bovine insulin. It is apparent that the reestablishment of the A^{20}–A^{21} amide moiety in the [des-A^{21}Asn]insulin analogues resulted in the reappearance of significant biological activity. The reduced binding affinity of these analogues as compared to that of the natural hormone is consonant with the recognized role of the A^{21} amino acid residue Asn as an important constituent of the binding region of insulin (Blundell et al., 1972; Blundell & Wood, 1975; Pullen et al., 1976). Interestingly, the isopropylamide analogue displays approximately 50% higher potency than the unsubstituted amide analogue described here and than the ethylamide analogue described in the following paper (Chu et al., 1987a); the isopropylamide group is sterically closer to the asparagine residue than are these moieties. The immunological potencies of both analogues were ca. 3-fold lower than their metabolic activity. This is not surprising since the C-terminal segment of the insulin A chain has been implicated as an important immunogenic determinant (Arquilla et al., 1969). From these findings, it appears reasonable to conclude that the A^{20}–A^{21} amide bond is involved in the expression of the biological activity of insulin (Chu et al., 1987b). It is of interest to note that the amide hydrogen of the A^{20}–A^{21} amide bond plays a role in maintaining the structure of insulin by hydrogen bonding with the carbonyl oxygen of the B^{23} Gly residue (Blundell et al., 1972). We have synthesized insulin A chain analogues lacking the A^{21} Asn residue, in which the A^{20} carboxyl group has been converted to a diethylamide or a dimethylamide group, each lacking the A^{20}–A^{21} amide hydrogen. These chain analogues, like the [des-A^{21}Asn]A chain previously mentioned, are unable to combine with the natural bovine B chain to produce the respective insulin analogues (unpublished data from this laboratory). It is thus apparent that the A^{20}–A^{21} amide hydrogen interaction with the B^{23} carbonyl oxygen is necessary for the proper orientation of the insulin A and B chains for combination to form insulin and for the stabilization of the insulin structure. This interaction may also play a role in ensuring the proper orientation of A^{20}–A^{21} amide bond within the insulin molecule for the expression of biological activity. In the following papers (Chu et al., 1987a,b), we present evidence indicating that this bond may be involved in modulating the biological activity of insulin independent of receptor binding affinity, thus implicating it as an important structural feature of the "message region" of insulin.

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Supplementary Material Available

Complete synthetic details of compounds I–VIII and Ia–VIIIa including references (12 pages). Ordering information is given on any current masthead page.

Registry No. I, 66960-27-8; I (NP ester), 53843-86-0; I (deblocked), 74201-66-4; Ia, 110456-85-4; Ia (deblocked), 110456-88-7; II, 110456-86-5; II (deblocked), 110456-90-1; IIa, 110456-87-6; IIa (deblocked), 110456-92-3; III, 110456-89-8; III (deblocked), 110456-94-5; III (TFA salt), 110457-18-6; IIIa, 110456-91-2; IIIa (deblocked), 110456-97-8; IIIa (TFA salt), 110457-19-7; IV, 110456-93-4; IV (deblocked), 110456-99-0; IV (TFA salt),

110457-20-0; IVa, 110456-96-7; IVa (deblocked), 110457-01-7; IVa (TFA salt), 110457-21-1; V, 110456-98-9; V (deblocked), 110457-03-9; V (TFA salt), 110457-22-2; V (azide precursor), 110434-00-9; V (hydrazide precursor), 42547-33-1; Va, 110457-00-6; Va (deblocked), 110457-05-1; Va (TFA salt), 110457-23-3; VI, 110457-02-8; VI (deblocked), 110457-07-3; VI (TFA salt), 110457-24-4; VI (azide precursor), 110434-03-2; VI (hydrazide precursor), 96573-60-3; VIa, 110457-04-0; VIa (deblocked), 110457-09-5; VIa (TFA salt), 110457-25-5; VII, 110457-06-2; VII (deblocked), 110457-11-9; VII (TFA salt), 110471-98-2; VII (hydrazide precursor), 96573-65-8; VII (azide precursor), 110456-72-9; VIIa, 110457-08-4; VIIa (deblocked), 110457-15-3; VIIa (TFA salt), 110457-26-6; VIII, 110457-10-8; VIII (deblocked), 110457-16-4; VIII (azide precursor), 110457-13-1; VIII (hydrazide precursor), 110457-12-0; VIIIa, 110457-14-2; VIIIa (deblocked), 110457-17-5; IX, 110471-96-0; IXa, 110471-97-1; i-$PrNH_2$, 75-31-0; L-Boc-Tyr(Bzl)-OH, 2130-96-3; L-Boc-Asn-ONP, 4587-33-1; L-Boc-Glu(Bzl)-ONP, 110456-95-6; β-chain sheep insulin (S-sulfonated), 18175-60-5; sheep [des-A^{21}-Asn,A^{20}-cysteinamide]-insulin, 110485-97-7; sheep [des-A^{21}-Asn,A^{20}-cysteinamide]insulin hydrochloride, 110486-00-5; sheep [des-A^{21}-Asn,A^{20}-cysteine isopropylamide]insulin, 110485-98-8; sheep [des-A^{21}-Asn,A^{20}-cysteine isopropylamide]insulin hydrochloride, 110486-01-6; insulin, 9004-10-8.

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