Studies on the Nature of Antigenicity of A and B Chains of Bovine Insulin*

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ABSTRACT: Antibodies to S-sulfonated A and B chains of bovine insulin have been produced in guinea pigs. The specificity of the antigen binding sites of the two antibodies has been investigated by comparing the inhibition of binding of ^{125}I-labeled homologous antigens to corresponding antibodies that is brought about with unlabeled antigens and with related unlabeled peptides from the same or different animal species. Beef, sheep, and pork A chains showed differences in their ability to inhibit binding of ^{125}I A chain (beef) by antibeef A-chain antibodies, suggesting that the portion of the chain wherein the differences in amino acid sequence (residues 8–10) for the three species occur constitutes at least part of the antigenic site. Antibodies to bovine B chain show no differences in their reactions with B chains of pork, sheep, and beef origin, all of which have identical amino acid structure. The B-chain antibodies do not react with A chains but do react with insulins. Here, differences were present in the reactions with beef, sheep, and pork insulins. Human insulin, which differs from pork insulin in that it contains threonine instead of alanine as the C-terminal residue in the B chain, reacted like pork insulin. The reactivities of desalanine pork B chain and intact pork B chain and of desalanine pork insulin and intact pork insulin were also alike. These results indicate that the C-terminal amino acid residue of the B chain is not involved in its antigenicity.

Antibodies to B chain reacted with desoctapeptide beef insulin (insulin lacking in the last eight amino acids of the C terminus of the B chain), although the inhibition of binding produced by the insulin derivative was much less than that obtained with the intact beef insulin. Fragments of B chain, *i.e.*, desoctapeptide B chain (B 1–22) and synthetic heptapeptide (B 23–29) either separately or together, do not react with anti-B-chain antibodies. These data have been interpreted to indicate that both amino acid structure and amino acid structure-induced conformation influence the antigen–antibody reaction.

The insulin molecule consists of two polypeptide chains connected by two disulfide bridges; a third disulfide bond is present as an intrachain disulfide bond on the A chain. Since the complete amino acid sequences of the insulins from several species are known, the insulins can serve as a set of model compounds for investigations of the influence of structural features on immunogenicity and on antigen–antibody interactions. In the present paper we report on the production of antibodies in guinea pigs against S-sulfonated A and B chains of bovine insulin and in their use to investigate the nature of antigenicity of the component chains and related peptides prepared from insulins of different species.

Materials and Methods

Bovine crystalline insulin (batch ML 74) was a product of Squibb and Sons. Pork crystalline insulin (lot PJ-5589), sheep crystalline insulin (lot PJ-4499), desalanine pork insulin (lot 516–621B–294), and human insulin (lot 516–734B–33) of Eli Lilly Co. were generous gifts from Drs. Mary A. Root and Ronald E. Chance. Desoctapeptide beef insulin (ES-III-R 53–10) and synthetic heptapeptide, B 23–29 (Gly-Phe-Phe-Tyr-Thr-Pro-Lys, see Figure 1) (IV-81-25) were generous gifts of Dr. F. H. Carpenter of the University of California, Berkeley, Calif.

Preparation of S-Sulfonated A and B Chains and Desoctapeptide S-Sulfonated B Chain. The S-sulfonated A and B chains from insulins of various species were prepared as described previously (Varandani, 1966). When the chains derived from smaller quantities of insulin (5–10 mg) were separated, the procedure was adapted as described in the legend of Figure 2. As an example, the separation of split products of one insulin (sheep) is shown. The purity of beef and pork chain preparations was established by complete amino acid analysis and also by electrophoresis at pH 1.9. In each case, complete amino acid analysis showed the presence of the correct amino acids in proper molar ratios and electrophoresis showed a single anion spot and a single cation spot corresponding to A chain and B chain, respectively. Because of the limited supply of material available the only criterion used to determine purity of the chains prepared from sheep and desalanine pork insulin was electrophoresis. Details of the methods have been described elsewhere (Varandani, 1966). Bovine crystalline zinc insulin was freed from zinc and

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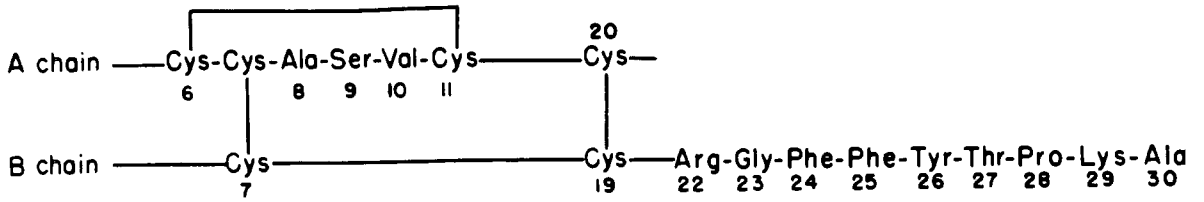

FIGURE 1: Schematic diagram of bovine insulin.

purified by treating with 0.2% EDTA in 8 M urea and chromatographing it on a Sephadex G-75 column with 50% acetic acid.

Desoctapeptide S-sulfonated beef B chain (B 1–22, see Figure 1) was prepared by subjecting S-sulfonated beef B chain to tryptic digestion at pH 9.4 (Young and Carpenter, 1961) and by purifying it by chromatographing it twice on a Sephadex G-75 column as described in Figure 2. Amino acid analysis showed the peptide possessed the expected amino acid composition (Table I) except for the presence of a trace of threonine, which should not affect the conclusions (see Results).

Preparation of ^{125}I-Labeled Antigens. ^{125}I as iodide in neutral solution, free from carrier iodide and reducing agents, was purchased from the Radiochemical Centre, Amersham. The iodination procedure of Greenwood *et al.* (1963) was employed except that for the separation of antigen from unreacted iodide the size of Sephadex G-25 column was 1 × 15 cm and the barbital buffer contained 0.2% bovine serum albumin. This yielded quantitative recoveries of labeled antigens.

TABLE I: Amino Acid Analysis of S-Sulfonated Desoctapeptide B Chain (B 1–22).[a]

Amino Acid	Number of Residues	
	Found	Theoretical
Aspartic acid	1.25	1
Threonine	0.04	0
Proline	0	0
Serine	1.10	1
Glutamic acid	2.82	3
Glycine	2.00	2
Alanine	1.17	1
Valine	3.04	3
Half-cystine	0.28	0
S-Sulfonated cysteine	*b*	2
Leucine	3.95	4
Tyrosine	0.95	1
Phenylalanine	1.18	1
Histidine	2.34	2
Arginine	1.07	1
Lysine	0	0

[a] The number of amino acid residues per molecule were calculated on the basis of glycine as 2. [b] *S*-Sulfocysteine is destroyed on acid hydrolysis yielding cysteine (Leach *et al.*, 1963), which may then be partly converted to cystine and possibly other products (Moore and Stein, 1963).

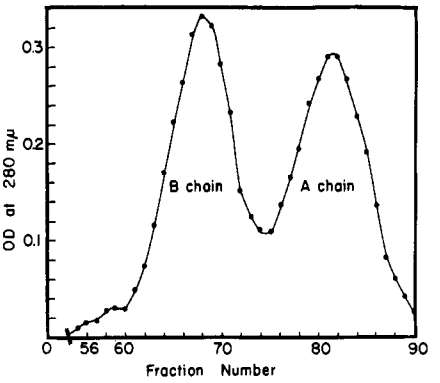

FIGURE 2: Separation of S-sulfonated A and B chains of sheep insulin on Sephadex G-75. Insulin (10 mg), dissolved in 0.4 ml of 0.2 M potassium phosphate, pH 7.5 buffer containing 8 M urea was treated with sodium sulfite (14 mg) and sodium tetrathionate (7 mg) at 37° for 3 hr. The volume was brought to 6 ml with the same buffer and the entire solution was applied to a 2 × 48 cm column of Sephadex G-75 which had been previously equilibrated with 50% acetic acid. The column was developed with 50% acetic acid at the rate of 5 min/fraction of 35 drops.

Preparation of Anti-A-Chain and Anti-B-Chain Sera. For the production of antisera, groups of three female guinea pigs were injected subcutaneously at 2-week intervals with 0.5 ml (0.6 mg of bovine insulin, 1 mg each of its S-sulfonated A chain or S-sulfonated B chain) of antigen solution mixed with an equal volume of Freund's complete adjuvant. The animals were bled by cardiac puncture 2 weeks after the third injection. Alternate immunization and bleeding were continued every 2 weeks. Antisera from a single animal from each group were used in the entire studies.

Detection of Antibody and Antibody–Antigen Reaction. These were carried out by the radioimmunological method of Skom and Talmage (1958). The procedure used was the same as that described by Morgan and Lazarow (1963) for the double-antibody immunoassay of insulin. All dilutions of standards and of sera were

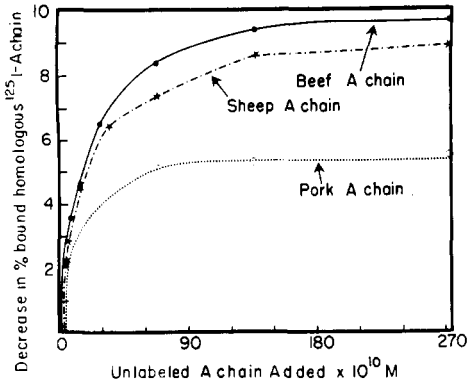

FIGURE 3: Inhibition of binding of homologous (beef) [^{125}I]S-sulfonated A chain to anti-S-sulfonated beef A-chain serum as a function of concentration of various unlabeled S-sulfonated A chains. Dilution of antiserum used was 0.01 and the amount of [^{125}I]S-sulfonated A chain was ~0.20 mμg/test. The binding of [^{125}I]S-sulfonated A chain in the absence of any unlabeled S-sulfonated A chain was about 11%. See text for details.

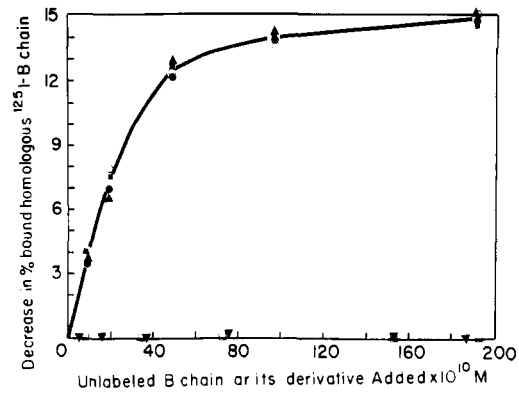

FIGURE 4: Inhibition of binding of homologous (beef) [^{125}I]S-sulfonated B chain to anti-S-sulfonated beef B-chain serum as a function of concentration of various unlabeled S-sulfonated B chains and their derivatives. Dilution of antiserum used was 0.04 and the amount of [^{125}I]S-sulfonated B chain was ~0.32 mμg/test. The binding of [^{125}I]S-sulfonated B chain in the absence of any unlabeled B chain was about 19%. See text for details. Beef B chain, ●; sheep B chain, ○; pork B chain, ▲; pork desalanine B chain, ■; beef desoctapeptide B chain (B1–22), ▼; and synthetic heptapeptide (B 23–29), □. For the sake of clarity, only one general curve has been drawn.

made with 0.15 M sodium chloride–borate buffer, pH 8.5, containing 5% bovine serum albumin fraction V (Nutritional Biochemical Co.). Mixtures which contained trace amounts of ^{125}I-labeled antigens in 0.1 ml and various concentrations of unlabeled antigen and related peptides from the same or different animal species in 1 ml were incubated with 0.1 ml of an appropriately diluted antiserum. The dilution of antiserum selected was one which had previously been determined to be still capable of binding the highest amount of homologous ^{125}I-labeled antigen used. The incubation periods of 1 hr at 25° and 16–20 hr at 5° were used. The tubes were then allowed to come to room temperature and were mixed with a sufficient amount of diluted normal guinea pig serum (0.1 ml) so that the total amount of guinea pig serum (normal and antiserum) in a total volume of 1.3 ml was equivalent to 0.1 ml of 0.01 guinea pig serum. This was followed by an addition of 0.1 ml (this amount was previously determined to be capable of producing maximum precipitation at 0.1 ml of 0.01 guinea pig serum) of rabbit antiguinea pig serum (Pentex, Inc., lot 12) to each tube. The tubes were then incubated for 1 hr at 25° and 2 hr at 5°. The precipitates were separated by centrifugation for 20 min at 5° and washed once with 0.5 ml of borate–sodium chloride buffer containing 1% bovine serum albumin. Radioactivity in the precipitates and in the combined supernatants was measured in a Packard well-type γ-ray spectrometer.

Results and Discussion

Antibodies to the immunizing antigen were produced by all the animals of each group. Control experiments in which normal serum and buffer were substituted for antiserum showed negligible amounts of reactions. Our results on the specificity of antibodies are in complete agreement with those reported recently by Yagi *et al.* (1965), so no details are presented. Antisera to S-sulfonated A chain are specific for S-sulfonated A chain; those to insulin[1] are specific for insulin; and antisera to S-sulfonated B chain react with S-sulfonated B chain and also with insulin.

In Figure 3 are shown the competitive inhibition of the binding of beef [^{125}I]S-sulfonated A chain to its antibodies by unlabeled S-sulfonated A chains from beef, sheep, and pork insulins. The inhibition with pork A chain was substantially less than that with beef A chain. The inhibition by sheep A chain was intermediate. The differences in the reactivity of the three A chains appear to be related to the number of amino acid residues in which they differ. Thus, beef and sheep A chains which differ in one amino acid residue (beef A chain contains serine in the ninth position, whereas sheep A chain contains glycine) reacted more alike than did beef and pork A chains, which differ in two amino acid residues (beef A chain contains alanine and valine, and pork chain contains threonine and isoleucine in the eighth and tenth positions, respectively (Harris *et al.*, 1956). In contrast to these results, the inhibition curves of binding of beef [^{125}I]S-sulfonated B chain to its

[1] Yagi *et al.* (1965) note that antibodies to insulin cross-reacted weakly with B chain when tested directly with ^{125}I B chain but did not observe any inhibition of the [^{125}I]insulin–antiinsulin system with unlabeled B chain.

antibodies with unlabeled beef, sheep, and pork S-sulfonated chains, all of which have the same amino acid structure, were similar (Figure 4).

The differences, seen with the A chains from beef, sheep and pork (Figure 3), are also seen, but in much smaller magnitude,[2] for the reactions of the insulins of these species to inhibit the binding of beef [^{125}I]S-sulfonated B chain to S-sulfonated B-chain antibodies (Figure 5). Insulins from various species show the same order of ability to inhibit as was observed with A chain antibodies (Figure 3), *i.e.*, beef insulin > sheep insulin > pork insulin. It is also interesting to note that human insulin, which differs from pork insulin in containing threonine instead of alanine as the C-terminal amino acid of B chain (Nicol and Smith, 1960), reacted like pork insulin; this indicates that the C terminal is not involved in the antigenicity. In this same connection, it will also be noted that the reactions of desalanine pork B chain[3] and desalanine pork insulin (lacking the C-terminal alanine residue) were similar to those of intact pork B chain and intact pork insulin, respectively (Figures 4 and 5). Antibodies to B chain reacted with desoctapeptide beef insulin (lacking the last eight amino acids of the C terminus of the B chain) though not as well as with intact insulin or intact B chain. They do not react at all with desoctapeptide S-sulfonated B chain (B 1–22)[4] or with heptapeptide (B 23–29). Addition of heptapeptide to desoctapeptide insulin or to desoctapeptide B chain did not restore the decrease or the loss of reactivity. It must be emphasized that all experiments have been performed with the same preparations of [^{125}I]S-sulfonated A chain and [^{125}I]S-sulfonated B chain, so the differences in reaction among the different species insulins and their A-chain preparations cannot be attributed to alterations of antigens during labeling.

These data, therefore, indicate that amino acid residues 8–10 of A chain represent at least part of the binding region of A chain and possibly constitute a site of antigenicity. There is a close resemblance in the inhibition curves between those obtained with B chain (Figure 4) and those obtained with insulin (Figure 5) as has also been observed by Yagi *et al.* (1965). Similar studies with antiinsulin antibodies and insulins of different species and desoctapeptide insulin have been carried out by Berson and Yalow (1959) and Yalow and Berson (1961). The differences in the reactivity reported by them with antiinsulin antibodies are in complete agreement with those observed in the present study with B-chain antibodies. It seems possible that the B chain might

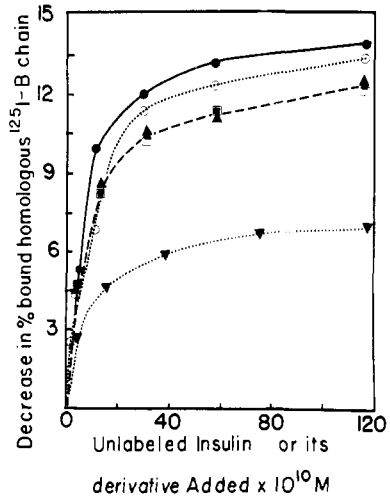

FIGURE 5: Inhibition of binding of homologous (beef) [^{125}I]S-sulfonated B chain to anti-S-sulfonated beef B-chain serum as a function of concentration of various unlabeled insulins and their derivatives. Dilution of antiserum used was 0.04 and the amount of [^{125}I]S-sulfonated B chain was ~0.32 mμg/text. The binding of [^{125}I]S-sulfonated B chain in the absence of any unlabeled insulin was 19%. See text for details. Beef insulin, ●; sheep insulin, ○; pork insulin, ▲; pork desalanine insulin, ■; human insulin, □; and beef desoctapeptide insulin, ▼. For the sake of clarity, for pork insulin, pork desalanine insulin, and human insulin which reacted alike only one general curve has been drawn.

have assumed a configuration similar to that of native insulin during the course of immunization.

The data presented show that the reactions of antigens (and cross-reacting polypeptides) with their antibodies depend both on their primary structure and their configuration. Thus, A chains of different primary structures react differently and B chains with identical primary structures reacted similarly (Figure 4). However, insulins of different species reacted differently and in a manner not entirely accounted for by the reactivities of the constituent chains, possibly because B chains assume different configurations when joined to A chains. This may also be inferred from the observations that the desoctapeptide insulin and not desoctapeptide B chain reacts with B-chain antibodies; the polypeptide configuration present in the original molecule of B chain must have been extensively altered by removal of octapeptide, and while desoctapeptide B chain still combined with A chain retains the configuration present in the original molecule. It is also possible that the entire octapeptide is involved in the antigenicity of B chain; the C-terminal acid residue alone does not seem to be involved. An influence of A chains in determining the configuration of the B chains in insulin might account for the differences between the reactivities of insulins of bovine, sheep, and pork origin; the behavior observed

[2] The differences are definite and are not due to experimental variations; the experiments were repeated several times and the differences were always present.

[3] For lack of sufficient material the A and B chains of human insulin could not be prepared for use in these studies.

[4] The preparation of desoctapeptide S-sulfonated B chain (B 1–22) showed a presence of trace amounts of threonine. Since the preparation did not compete against the binding of [^{125}I]S-sulfonated B chain by anti-S-sulfonated B-chain antibodies even when 3.2×10^{-6} M was used, the presence of threonine would not alter the conclusions.

with the separated A chains is consistent with the differences found with the intact insulins. These observations offer no explanation for the reported difference in reactivities of pork and human insulins, whose only structural difference is the noncritical C-terminal amino acid (Berson and Yalow, 1963).

Relationships such as shown in Figures 3 and 4 can be used for assaying A chain and B chain. Insulin and A chain may be measured directly using antiinsulin and anti-A-chain antibodies, respectively. The amount of B chain may be determined as the difference between the total inhibition of binding of ^{125}I B chain by anti-B-chain antibodies due to both B chain and insulin and the inhibition corresponding to amount of insulin (determined from the standard curve, ^{125}I-B-chain–anti-B-chain antibodies *vs.* unlabeled insulin system). Preliminary studies, carried out with mixtures of beef A chain, beef B chain, and beef insulin in the buffer system, indicate that it is possible to measure them in the presence of each other in this manner. From the similarity in the reaction between human insulin and pork insulin and its intact and desalanine component chains[3] (Figure 4), it appears that the pork antibodies can probably be used to measure the A and B chains of material of human origin. An abstract reporting the use of antibodies to assay A and B chains is on record (Meek *et al.*, 1966).

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