Reactions of Proinsulin and its Derivatives with Antibodies to Insulin (34966)

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It has been repeatedly demonstrated that proinsulin can compete successfully with radioiodinated insulin for sites on antibodies in guinea pig anti-insulin serum, and that it does so less effectively than insulin itself (1). It has also been shown that the abilities of various species of insulin to compete in this system can vary widely with the species of unlabeled insulin used and the particular specimen of anti-insulin serum to which they are exposed (2). In the present studies, reactions between insulin antibodies and various derivatives of proinsulin were studied in two systems in an attempt to distinguish between the antigenic specificities of insulin and proinsulin.

Materials and Methods. Two contrasting procedures were adopted and both have been described in detail elsewhere (3, 4):

(i) *Method B* (back-titration). A constant volume of buffered solution (0.1 ml) containing anti-insulin serum (GPAIS) was *preincubated* at 36° for 60 min with varying amounts of unlabeled antigen (insulin, proinsulin) dissolved in the same buffered solution (0.2 ml). The products were then *incubated* for 30 min at room temperature (23–28°) with a solution (0.1 ml) containing radioiodinated (^{125}I-procine or ^{131}I-bovine) insulin with or without additional unlabeled insulin of the same species.

(ii) Method C (conventional). The solutions containing unlabeled antigen (0.2 ml) and the radioiodinated insulin (0.1 ml) were first thoroughly mixed and then *incubated* at room temperature for 30 min with the solution (0.1 ml) containing anti-insulin serum.

Following incubation in either system, labeled insulin bound to antibodies in the GPAIS was *precipitated* with alcohol (1.6 ml; 95% v/v) at room temperature. The precipitates, after repeated agitation during 30 min at room temperature, were deposited by centrifugation, washed once with alcohol (2.0 ml; 76%, v/v), and their γ-radioactive contents were measured in an automatic γ-counter (Series 3000, Model 5052; Packard Instruments Company, La Grange, Illinois).

All solutes were dissolved in a buffered solution (0.05 *M*, Na_2 HPO_4/KH_2PO_4; pH 7.0) containing sodium chloride (0.4%, w/v) and normal guinea pig serum (3%, v/v; Hyland Laboratories, Los Angeles, California). Dissolved in this solution (3% NGPS), as required, were:

(i) Guinea pig anti-insulin serum (GPAIS). Antibovine (Lot No. 468) and antiporcine (Lot No. 515) insulin sera were prepared by methods described elsewhere (5, 6).

(ii) Unlabeled antigens. These included porcine (Lot No. 615-984B-99-C) and bovine (Lot No. 615-984B-122C) *proinsulin;* the *Desnonapeptide of porcine proinsulin* (Lot No. 615-1039B-202-2); the *Desoctapeptide of porcine insulin* (Lot No. 258-1011B-49-3); the *A* (Lot No. 258-970B-142-8) and *B* (Lot No. 258-1011B-10-1) *chain peptides of porcine insulin*; and the connecting or *C peptide in porcine proinsulin* (Lot. No. 615-984B-81-F2). All these compounds were isolated, purified, and identified by Dr. R. A. Chance and kindly provided by Dr. W. Bromer of Eli Lilly and Company, Indianapolis, Indiana. Unlabeled bovine (Lot PJ4609, 23.6 ± 1.7 U/mg) and porcine (Lot PJ5589, 21.7 ± 1.1 U/mg) *insulins* were also donated by Eli Lilly and Company. Stock solutions of insulins (100 U/ml in 0.6% acetic acid, v/v) were kept at 0 to 4° and diluted immediately before use in 3%

NGPS. The derivatives of insulin and proinsulin were provided as powders or in solution and were diluted in 3% NGPS for use within 3 days.

(iii) Radioiodinated insulins. Specimens of ^{131}I-bovine (5–12 mCi/mg; Abbott Laboratories, North Chicago, Illinois) and ^{125}I-porcine (ca. 100 mCi/mg; Cambridge Nuclear Corporation, Cambridge, Mass.) insulins were obtained commercially and were assumed to have the potencies (mg of insulin/ml) stated by the manufacturers. They were diluted for experimental use in 3% NGPS on the assumption that they had biological potencies of 25 U/mg.

The proportion (percent) of added labeled insulin bound under the various experimental conditions was calculated from the expression:

$$y_c = 100\,(C_s - C_{Bl})/(C_{Exc} - C_{Bl}), \quad (1)$$

where C_{Exc}, C_s, and C_{Bl} are the counts of radioactivity measured in tubes containing (respectively) an excess of anti-insulin serum, the sample under investigation, and the blank solution with no added anti-insulin serum. It can also be shown that this percentage of added labeled insulin bound by antibodies (y_c) can be correlated with the amount of unlabeled antigen in the system (x) according to the conditions used:

(a) Method B (back-titration).

$$y_c = m_c x + C_c, \quad (2)$$

where m_c is the regression coefficient and C_c the intercept of the regression line ($y = 0$).

(b) Method C (conventional).

$$C_c/y_c = 1 + Mx, \quad (3)$$

where C_c is again the amount of labeled insulin bound during incubation when there is no added unlabeled antigen; and M is a regression coefficient.

The derivations of these two equations have been discussed and described in detail elsewhere (3). Significant differences between

TABLE I. Regression Coefficients Obtained by Methods B ($m_c \pm S_b$) and C ($M \pm S_b$) with the Use of Antiporcine Insulin Serum, Labeled Porcine Insulin, and the Indicated Unlabeled Antigens.

The coefficients are stated on an absolute and molar basis for each antigen, and those which were not significantly different from zero are marked with ([a]). The number of individual points used to calculate each regression coefficient is shown in parentheses. Further experimental details are given in the text.

	Back-titration ($m_c \pm S_b$)		Conventional assay ($M \pm S_b$)	
Antigen (mol wt)	Absolute (μU insulin/mμg of preincubated antigen)	Molar (μU insulin/μμmole of preincubated antigen)	Absolute (C_c/y_c/mμg of antigen)	Molar (C_c/y_c/μμmole of antigen)
Proinsulin (porcine) (9100)	−8.70 ± 0.29 (44)	−79.12 ± 2.62	0.0355 ± 0.0007 (33)	0.3230 ± 0.0063
(bovine) (8700)	−5.27 ± 0.60 (31)	−45.82 ± 5.20	—	—
desnonapeptide (8100)	−9.23 ± 0.34 (31)	−74.79 ± 2.74	0.0631 ± 0.0010 (33)	0.5111 ± 0.0081
Insulin (porcine) (5700)	−17.42 ± 0.42 (108)	−99.28 ± 2.38	0.1210 ± 0.0015 (33)	0.6897 ± 0.0085
desoctapeptide (4900)	−6.15 ± 0.28 (43)	−30.14 ± 1.39	0.0160 ± 0.0021 (33)	0.0784 ± 0.0102
A chain (2700)	−0.22 ± 0.21[a] (33)	—	—	—
B chain (3600)	−0.52 ± 0.31[a] (33)	—	—	—
C peptide (3000)	−0.23 ± 0.15[a] (32)	—	0.0013 ± 0.0007[a] (32)	—

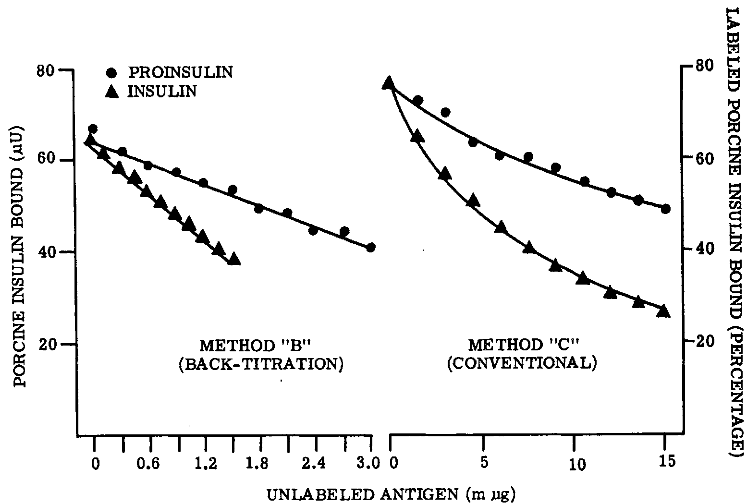

FIG. 1. A comparison of labeled porcine insulin binding by guinea pig anti-insulin serum during incubation at room temperature for 30 min (a) in the presence of unlabeled porcine insulin or proinsulin (Method C); and (b) subsequent to preincubation at 36° for 1 hr with unlabeled porcine insulin or proinsulin (Method B). More experimental details are given in the text.

regression coefficients were demonstrated by statistical methods described by Snedecor and Cochran (7).

Results. In two series of experiments the reactions of proinsulin and its derivatives with guinea pig anti-insulin serum were compared. The results are summarized in Table I and Figs. 1 and 2.

(i) Method B (back-titration). Unlabeled antigens (0 to 3.0 mμg/0.2 ml) were preincubated with GPAIS (25 mμl, Lot No. 515/0.1 ml) and then incubated with a solution (0.1 ml) containing unlabeled (90 μU) and labeled (ca. 10 μU) porcine insulin. The results obtained in three individual experiments with preincubated porcine insulin were not significantly different from one another and have been combined in the result shown in Table I. The regression coefficients (m_c) obtained after preincubation of A chain, B chain and the connecting C peptides were not significantly different from zero ($p>0.100$). As shown in detail in Table I, the regression coefficients (stated in terms of absolute amounts of preincubated antigen) for porcine proinsulin and its desnonapeptide were not significantly different from one another ($p>0.100$) but both were significantly less than that for porcine insulin ($p<0.001$) and greater than that for the desoctapeptide of porcine insulin ($p<0.005$). When results were expressed on a molar basis (Table I), these differences persisted. Compared with the

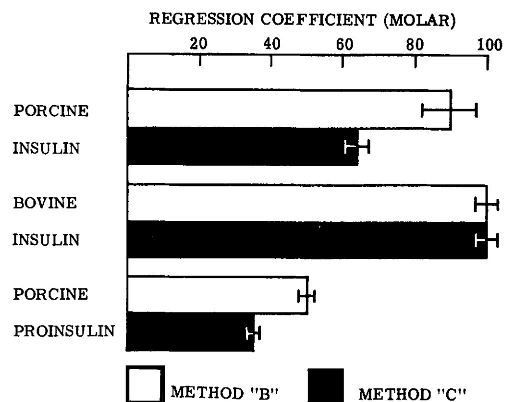

FIG. 2. A comparison of regression coefficients obtained by methods B (m_c) and C (M) when the unlabeled antigens were bovine insulin, porcine insulin and porcine proinsulin and the other two reagents were labeled bovine insulin and antibovine insulin serum. For purposes of comparison, the regression coefficients derived according to Eq. (2) and (3) are expressed as percentages of those found with unlabeled bovine insulin (100%). Further experimental details are given in the text.

regression coefficient stated on this basis (μU of insulin/$\mu\mu$mole of preincubated antigen) for porcine insulin (100%), those for porcine proinsulin and its desnonapeptide (80 and 75%) were lower but not significantly different from each other ($p>0.250$); and that for the desoctapeptide of insulin was even smaller. The regression coefficient for porcine proinsulin was significantly greater than that of bovine proinsulin ($p<0.005$) whether stated in absolute terms or on a molar basis (Table I).

In a second series of experiments carried out over several months, the same range of weights (0 to 2.0 mμg/0.2 ml) of porcine insulin, bovine insulin, and porcine proinsulin were preincubated with the same volume of the same batch of GPAIS (20 mμl Lot No. 468/0.1 ml) and then incubated with different batches of labeled bovine insulin (ca. 70 μU/0.1 ml). When compared with the mean regression coefficient ($\pm$ SEM) obtained after preincubation of bovine insulin (taken as -100 ± 2.30; 15 expts.), that for preincubated porcine insulin (-90.2 ± 7.30; 8 expts.) was not significantly different ($p>0.100$) but that for porcine proinsulin (50.8 ± 2.10; 10 expts.) was substantially lower ($p<0.005$); this comparison is made on a molar basis (Fig. 2).

(ii) Method C (conventional). In a single experiment, the various antigens (0 to 15 mμg/0.2 ml) were mixed with a solution (0.1 ml) containing unlabeled (90 μU) and labeled (ca. 10 μU) porcine insulin and then incubated with GPAIS (30 mμl, Lot No. 515; 0.1 ml). The regression coefficient obtained with the C peptide as antigen was not significantly different from zero ($p>.100$). Other results, summarized in Table I and illustrated in Fig. 1, show that compared with porcine insulin (100%), porcine proinsulin (47%), its desnonapeptide (74%), and the desoctapeptide (11%) compete much less successfully with the homologous labeled insulin for sites on the insulin antibodies in GPAIS.

When the system included antibovine insulin serum (35 mμl Lot No. 468/0.1 ml), labeled bovine insulin (ca. 100 μU/0.1 ml) and unlabeled antigens (0 to 10 mμg/0.1 ml), the regression coefficient obtained with bovine insulin (taken as $+ 100 \pm 2.60$; 44 points in one expt.) was significantly greater ($p<0.001$) than those found for porcine insulin (64 ± 2.5; 84 points in two experiments) and porcine proinsulin (35 ± 1.2; 84 points in two experiments); these comparisons were made on a molar basis (Fig. 2).

Discussion. It is now well known that proinsulin is capable of reacting with insulin antibodies in guinea pig anti-insulin serum (1, 8–12). This is confirmed by the present findings which also show that the behaviors of insulin, proinsulin, and their derivatives are dependent upon the immunological system in which they are tested.

In the "conventional" assay system (Method C) used by other investigators, unlabeled antigen and labeled insulin are simultaneously exposed in excess to the insulin antibodies, and so compete with each other for active sites on those antibodies. Under these conditions, as illustrated in Fig. 2, both porcine proinsulin (35%) and porcine insulin (64%) compete less effectively with labeled bovine insulin than does unlabeled bovine insulin (100%) for sites on antibodies to bovine insulin; for comparative purposes the regression coefficient obtained with unlabeled bovine insulin, as shown in the text, is taken as 100%. When the system includes labeled porcine insulin and antibodies to porcine insulin (Table I), unlabeled porcine insulin (100%) competes about twice as effectively as porcine proinsulin (47%). Loss of immunological activity is particularly noticeable when the insulin molecule is disrupted, as in the desoctapeptide of insulin (11%). From these observations it can be concluded that even small changes in structure of the antigen, such as those seen in the different species of insulin (2), can result in quite marked alterations in immunoreactivity in this competitive system.

In the "back-titration" system (Method B), unlabeled antigen is first exposed to an excess of insulin antibodies, and only after these have had time to react are the residual free sites of reaction on the antibodies exposed during incubation to the labeled insulin. Thus reactive antibodies are never ex-

posed simultaneously to both the antigen and the labeled insulin and no competitive reaction is therefore involved. Under these conditions we have already shown (4, 13, 14) and here confirm (Fig. 2), that it is not possible to distinguish between several mammalian species of insulin (*e.g.*, bovine, porcine, rat, sheep, and human) having very similar amino-acid sequences. This is in marked contrast to the effects observed here after preincubation of anti-insulin sera with derivatives of porcine and bovine insulin and proinsulin (Table I and Fig. 2). The presence of the C peptide in porcine proinsulin (80%) and its desnonapeptide (76%) resulted in moderate reduction of immunoreactivity compared with that of porcine insulin itself (100%); the regression coefficient obtained after preincubation with insulin is taken as 100% for purposes of comparison. When the nature of the C peptide is changed, as it is in bovine proinsulin (1), reactivity is further reduced (47%). These results suggest that the C peptide in the proinsulin molecule covers reactive sites on the insulin molecule and thus reduces the absolute ability of the antigen to react with antibody sites in anti-insulin serum. Differences in structure of this chain of amino acids could alter ability to mask these sites and might thus be responsible for the differing reactivities of bovine and porcine proinsulin (Table I).

In conclusion, we infer from the results presented here that insulin has more than one active immunological site of reaction; that these could not be demonstrated by the "conventional" method of assay; and that some of them are masked by the C chain of amino acids present in the proinsulin molecule.

Summary. The reactions of porcine proinsulin and its degradation products with insulin antibodies in guinea pig anti-insulin serum can be shown to vary with the immunological system used for study. From the results obtained with two such systems it is concluded that the connecting chain of amino acids in the molecule of proinsulin must mask sites on the insulin molecule which are capable of reacting with insulin antibodies.

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