

Familial hyperproinsulinemia: Partial characterization of circulating proinsulin-like material*

(proinsulin intermediate/immunoabsorption/genetic defect/B-chain/C-peptide)

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ABSTRACT Familial hyperproinsulinemia is an autosomal dominant defect that is associated with strikingly elevated levels of serum proinsulin-like material. Our studies show that trypsin converts familial hyperproinsulinemia proinsulin to insulin more slowly than it converts a ¹³¹I-labeled porcine proinsulin marker. Molar yields of insulin indicated that the material may be an intermediate proinsulin. Studies with two human C-peptide antisera that differ in their relative immunoreactivity with human C-peptide and proinsulin showed that the two antisera reacted equally with familial hyperproinsulinemia proinsulin, suggesting that it is a partially cleaved proinsulin intermediate. Sulfitolysis of highly purified material to break the inter- and intra-chain disulfide bridges and subsequent adsorption on a specific B-chain antibody covalently bound to Sepharose beads showed that the C-peptide was still connected to the B-chain. These data indicate that familial hyperproinsulinemia proinsulin is normally cleaved at the C-peptide-A-chain linkage site. A structural abnormality appears to underlie familial hyperproinsulinemia proinsulin, which impairs its cleavage at the B-chain-C-peptide linkage site.

Familial hyperproinsulinemia (FH) is a genetic defect (1) that is associated with strikingly elevated levels of serum proinsulin-like material (PLM). The PLM constitutes a major fraction (74-91%) of the total circulating immunoreactive insulin in both the fasted and stimulated states. The defect is present in members of four generations of the kindred, ranging in age from 6 months to 73 years, and is transmitted as an autosomal dominant trait. The affected progeny are asymptomatic with no apparent increase in susceptibility to hyperglycemia or the development of diabetes mellitus, suggesting that the high circulating levels of PLM contribute to the maintenance of normal carbohydrate tolerance.

We report the partial characterization of the circulating PLM in familial hyperproinsulinemia, which demonstrates that the PLM is a partially cleaved proinsulin intermediate. The structural abnormality resides at the cleavage site linking the B-chain to the C-peptide and impairs the complete conversion of familial hyperproinsulinemia proinsulin to insulin within the beta cells of affected individuals.

METHODS

Patient Material. Plasma (400-500 ml) was obtained by plasmapheresis from two males and one female with the defect (II-9, III-8, and III-29 in ref. 1). The study was approved by the Clinical Investigation Committee at Children's Hospital Medical Center, and informed consent was obtained.

The levels of PLM and insulin in samples were determined by column chromatography. In the trypsin conversion studies, plasma (0.5 ml) was applied to a 1 × 50 cm column of Bio-Gel

P-30 (Bio-Rad), equilibrated in borate/bovine serum albumin (0.5%) (BBSA) buffer, pH 8.0, to separate the PLM and insulin. Fractions (1 ml) were collected and immunoreactive insulin in each was determined by radioimmunoassay. Larger quantities of FH-PLM were prepared by chromatography of 100 ml of plasma on a 5 × 100 cm column of Sephadex G-50, fine (Pharmacia), equilibrated in 0.05 M ammonium carbonate, pH 7.9. Fractions of 10 ml were collected and 1-ml aliquots were assayed for immunoreactive insulin. The FH-PLM peak was pooled, lyophilized, and stored at -30°C until use.

Highly purified FH-PLM was prepared by extraction of the partially purified and lyophilized FH-PLM region (from a 100-ml column run) with 2 mM HCl. The extract was lyophilized and redissolved in 20 ml of BBSA buffer and recirculated through a 0.5-ml column of guinea pig anti-insulin globulin coupled to Sepharose 4B beads (see below). Approximately 90% of the FH-PLM was bound to the antibody-Sepharose during a 5-hr recirculation at room temperature. The beads were washed with 50 ml of phosphate buffered saline, and the FH-PLM was eluted from the beads with 0.1 M glycine/0.05 M HCl. The glycine was removed by chromatography of the eluate on a 1 × 25 cm column of Bio-Gel P-2, equilibrated with 0.05 M ammonium carbonate buffer. The purified FH-PLM was lyophilized and stored for use in subsequent studies.

Trypsin Conversion Studies. Conversion of purified porcine proinsulin was studied by incubation of 10-ng samples of porcine proinsulin and ¹³¹I-labeled porcine proinsulin (porcine ¹³¹I-proinsulin) (20,000 cpm) in 0.5 ml of 0.1 M Tris-HCl, pH 8.3/2 mM NaCl (Tris/NaCl buffer) containing 0.1% albumin. The mixture was shaken in a 37°C waterbath, appropriate dilutions of a stock solution (1.0 mg/ml) of diphenyl carbamyl chloride-treated trypsin (Sigma) were added to each sample, and the incubation was allowed to proceed for various time periods. The tryptic action was stopped by placing the samples on ice and adding 100-fold excess of soybean trypsin inhibitor (Worthington). The conversion of PLM in the patient's plasma was performed by adding trypsin directly to 0.5 ml of plasma containing known amounts of PLM and insulin (determined by column chromatography and radioimmunoassay) and 20,000 cpm of porcine ¹³¹I-proinsulin. The conversion of the partially purified lyophilized FH-PLM was studied by dissolving known amounts of lyophilized FH-PLM and 20,000 cpm of porcine ¹³¹I-proinsulin in 0.5 ml of Tris/NaCl buffer, and then adding various amounts of trypsin to the mixture. After addition of the trypsin inhibitor, the reaction mixture was chromatographed on a 1 × 50 cm Bio-Gel P-30 column and 1-ml fractions were collected. The ¹³¹I radioactivity in each fraction was counted, and the percentages of counts in the

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Abbreviations: FH, familial hyperproinsulinemia; PLM, proinsulin-like material; BBSA, borate/bovine serum albumin.

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proinsulin and insulin peaks were then calculated to determine porcine ^{131}I -proinsulin conversion to insulin. Each fraction was subsequently assayed for immunoreactivity by using ^{125}I -insulin as a tracer, and the amounts of proinsulin and insulin were determined by using human insulin and proinsulin standards. The amount of proinsulin or PLM converted was calculated as the difference between pre- and post-incubation levels in the samples as determined by column chromatography. Because insulin (M_r 6000) represents approximately 66% of the mass of a proinsulin molecule (M_r 9000), the molar yield of insulin was calculated by the following formula:

$$\text{Molar yield of insulin} = \frac{\text{Net yield of insulin (ng)}}{\text{Converted proinsulin (ng)} \times 0.66}$$

Sulfitolysis. Intact porcine proinsulin (2 μg), porcine desdipeptide (Lys₆₂-Arg₆₃ absent) proinsulin (5 μg), and highly purified FH-PLM (approximately 40 ng estimated against the human proinsulin standard) were sulfonated by the following method.

A solution of 8 M urea (Schwarz/Mann; Ultra Pure) was passed through a 10-ml packed column of Amberlite MB-3 ion exchange resin (Mallinckrodt). Sufficient amounts of potassium phosphate salts were then added to make an 8 M urea/0.05 M potassium phosphate buffer, pH 7.5. The respective proinsulin materials were lyophilized in test tubes, and 7.0 mg of sodium tetrathionate (kindly prepared by Clyde Zalut, Division of Immunology, Children's Hospital Medical Center, Boston, MA) and 14 mg of sodium sulfite were added. Urea buffer (400 μl) was added, and the mixture was shaken until dissolved and then incubated for 3 hr in a 37°C waterbath. The reaction mixture was then chromatographed on a 1 \times 25 cm Bio-Gel P-2 column equilibrated with 0.05 M ammonium carbonate, and 0.9-ml fractions were collected. The fractions corresponding to the total elution volume of blue dextran were collected and lyophilized for use in subsequent studies.

Coupling of Antibodies to Sepharose Beads. Guinea pig anti-bovine insulin serum was kindly donated by Peter Wright (Indiana Univ. School of Medicine, Indianapolis, IN) (Lot 570, 50% binding capacity = 1.3 units of insulin per ml). An equal volume of cold, saturated ammonium sulfate solution was added to 0.5 ml of serum, and the resulting globulin precipitate was separated by centrifugation, redissolved, dialyzed, and used for binding to Sepharose 4B beads. The anti-insulin globulin (9.6 mg) was coupled to an 8-ml gel bed volume of cyanogen bromide-activated Sepharose 4B beads and the mixture was stirred overnight at 4°C. Glycine (20 mM) was then added to react with the remaining activated Sepharose bead sites. The beads were then washed extensively with phosphate-buffered saline and stored in phosphate-buffered saline containing 0.01% merthiolate, penicillin, and streptomycin. Guinea pig anti-bovine insulin B-chain antibody was coupled directly to cyanogen bromide-activated Sepharose 4B beads by using 0.1 ml of the antiserum and a 10-ml gel bed volume according to the above method.

Radioimmunoassays. Insulin immunoreactivity was measured by a modification (2) of the double antibody method of Morgan and Lazarow (3). Two guinea pig anti-bovine insulin sera, which were gifts from Lillian Recant and Peter Wright (Lot 496), were used in the assays. The two antisera are virtually identical in their properties and immunoreactivity with human and porcine proinsulin and insulin. Human insulin is 4–5 times more immunoreactive than human proinsulin on a molar basis in the assay system used, and standard curves describing their relative immunoreactivity were published (2). Similar relative molar ratios of immunoreactivity exist between porcine insulin and proinsulin. Intermediate porcine proinsulins [desdipeptide

proinsulin, desnonapeptide (C₅₅₋₆₃ absent) proinsulin, and "split" (Leu₅₄-Ala₅₅ split) proinsulin] are intermediate in their immunoreactivity in this assay system, being 2–2.5 times more immunoreactive than intact porcine proinsulin. Intermediate human proinsulins are not available for direct comparison with human insulin and proinsulin.

Guinea pig anti-bovine insulin B-chain serum was a gift from P. T. Varandani (Fels Research Institute, Yellow Springs, OH) (Lot VI-p45-15-27). Characterization of this antibody to the B-chain of insulin is presented in *Results*. The radioimmunoassay for B-chain was performed by the same double antibody immunoassay used for insulin with the antibody used at a final dilution in the assay tubes of 1:180,000. Pure S-sulfonated bovine B-chain was used to prepare standards as well as ^{125}I -B-chain label.

C-peptide immunoreactivity was determined by the double antibody radioimmunoassay technique as described (4). For certain studies, two guinea pig anti-human C-peptide sera were used that differed in their respective immunoreactivity to human proinsulin. Antiserum Y reacted with human proinsulin $1/3\text{rd}$ as well as with C-peptide, and antiserum M 1230 reacted with proinsulin $1/10\text{th}$ as well as with C-peptide.

Peptides were iodinated by a modification (5) of the chloramine-T method developed by Hunter and Greenwood (6). ^{125}I and ^{131}I were obtained from Industrial Nuclear Corporation (St. Louis, MO). Porcine proinsulin (Lot 615-1112B-278), porcine desdipeptide proinsulin (Lys₆₂-Arg₆₃ absent, Lot 615-1112B-32), porcine desnonapeptide proinsulin (C₅₅₋₆₃ absent, Lot 615-1112B-31), and porcine "split" proinsulin (Leu₅₄-Ala₅₅ bond cleaved, Lot 615-1071B-30-2) were a gift from Ronald E. Chance of Eli Lilly.

RESULTS

Trypsin Conversion Experiments. Porcine proinsulin, familial hyperproinsulinemia plasma, and partially purified FH-PLM were stable on incubation in the absence of trypsin, and no conversion of immunoreactive proinsulin to insulin or of ^{131}I -proinsulin to ^{131}I -insulin was observed. These incubations were performed with each of the following experiments.

Porcine proinsulin conversion. Porcine proinsulin (10 ng) and 20,000 cpm of porcine ^{131}I -proinsulin were incubated with trypsin (0.5 $\mu\text{g}/\text{ml}$) for 2, 4, 10 and 15 min and then subjected to gel filtration (Fig. 1 *top*). At each time point, the extent of conversion of immunoreactive porcine proinsulin to insulin was similar to the extent of conversion of porcine ^{131}I -proinsulin to ^{131}I -insulin. The molar yield of insulin at each time period corresponded to the amount of converted proinsulin material and averaged 0.89—i.e., the conversion was approximately stoichiometric.

Familial hyperproinsulinemia plasma conversion. FH plasma (0.5 ml) containing known amounts of FH-PLM and insulin was incubated with 20,000 cpm of ^{131}I -proinsulin and trypsin (20 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$) for various time periods followed by gel filtration (Fig. 1 *middle*). The conversion of FH-PLM present in plasma was markedly retarded as compared to the conversion of porcine ^{131}I -proinsulin. The molar yield of insulin at the various time points averaged approximately one-half of that expected from the amount of converted FH-PLM (mean molar yield = 0.58).

Partially purified FH-PLM conversion. FH-PLM (estimated against a human intact proinsulin standard) and 20,000 cpm of ^{131}I -proinsulin were incubated with trypsin followed by gel filtration (Fig. 1 *bottom*). Again, the conversion of partially purified FH-PLM was considerably slower than the conversion of porcine ^{131}I -proinsulin. With increasing concentrations of trypsin or longer incubation times, FH-PLM can be completely converted to insulin—e.g., 90% conversion at a trypsin con-

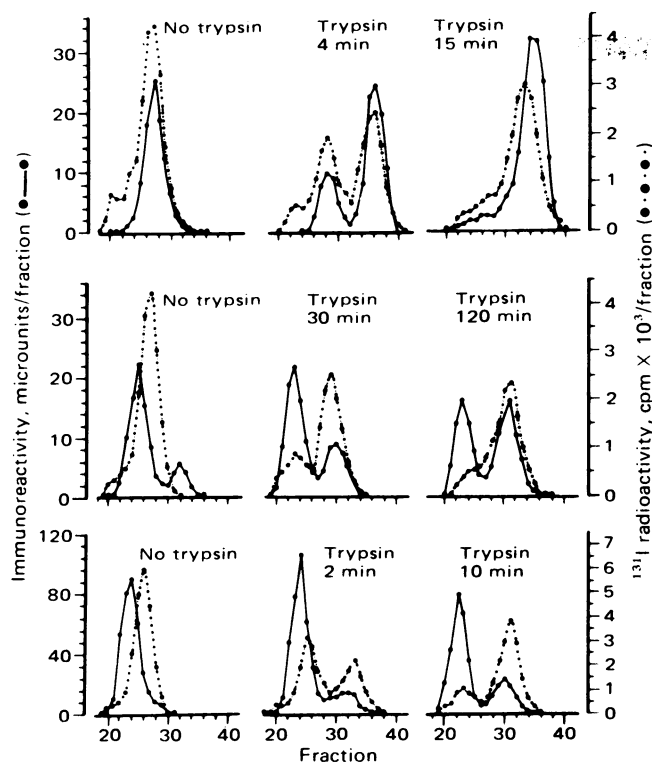


FIG. 1. Trypsin conversion studies. Porcine proinsulin (Top), FH plasma (Middle), and purified FH-PLM (Bottom) were incubated with porcine ¹²⁵I-proinsulin (•••••), with and without trypsin for the time periods indicated. FH-PLM (—) was converted to insulin by trypsin at a slower rate than the porcine proinsulin marker. Trypsin was at 0.5 μg/ml (Top), 50 μg/ml (Middle), and 1 μg/ml (Bottom).

centration of 5 μg/ml and a 60-min incubation period and 94% conversion at 25 μg/ml and 15 min. The molar yield of insulin in these experiments again averaged one-half of that expected (mean molar yield = 0.53).

These experiments indicate that FH-PLM can be converted to insulin by trypsin, albeit more slowly than intact porcine proinsulin. The conversion was time- and trypsin concentration-dependent. The approximate 50% molar yield of insulin from FH-PLM in these experiments suggests that the amount of FH-PLM (originally calculated from a human intact proinsulin standard) was overestimated approximately 2-fold. These findings are consistent with the hypothesis that FH-PLM is an intermediate proinsulin and with the fact that the use of human intact proinsulin as a standard in the radioimmunoassay is inappropriate in estimating the actual content of proinsulin in familial hyperproinsulinemia.

Immunoreactivity of FH-PLM with C-Peptide Antisera. Familial hyperproinsulinemia plasma (2 ml) was chromatographed on a 1.5 × 75 cm Bio-Gel P-30 column equilibrated with 3 M acetic acid to separate the PLM and C-peptide present in the plasma. Aliquots of each fraction were dried under reduced pressure and assayed for C-peptide immunoreactivity with two antisera to human C-peptide (Fig. 2).

Due to the high PLM content of familial hyperproinsulinemia plasma, the PLM constitutes a larger proportion of the total C-peptide immunoreactivity than in normal subjects. As expected, both antisera reacted equally with the C-peptide peak. However, the two antisera that have different reactivity with intact human proinsulin reacted equally with FH-PLM. In normal sera, the PLM component reacts much less well with one antiserum than the other. These results suggest that the PLM in familial hyperproinsulinemia may indeed be a two-chain proinsulin intermediate rather than an intact single-chain proinsulin.

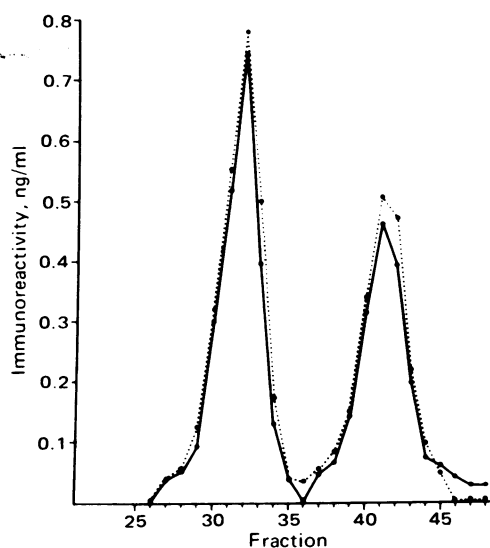


FIG. 2. Bio-Gel P-30 column chromatography of 2 ml of familial hyperproinsulinemia plasma. The FH-PLM (first peak) and C-peptide (second peak) were assayed with two antisera to human C-peptide that differ in their relative immunoreactivity with human proinsulin. Note identical immunoreactivity of FH-PLM peak with both antisera. —, Antiserum Y; •••••, antiserum M 1230.

Localization of Structural Defect in Familial Hyperproinsulinemia Proinsulin. The previous experiments suggested that FH-PLM may be a two-chain proinsulin intermediate cleaved at one linkage site, and possessing a structural defect at the other linkage site rendering it less susceptible to trypsin cleavage. The two cleavage sites in proinsulin are between the Arg-Gly residues connecting the C-peptide to the A-chain and the Arg-Glu residues connecting the B-chain to the C-peptide. A strategy was evolved to localize the defective cleavage site, based on the fact that the two chains in intermediate proinsulin (like insulin) are held together by disulfide bridges. Sulfitolysis of highly purified FH-PLM to break the disulfide bridges would result in two chains with the following possible alternate compositions: (i) an A-chain-C-peptide fragment and a free B-chain, or (ii) a B-chain-C-peptide fragment and a free A-chain. These two possible outcomes can be distinguished by adsorption of the S-sulfonated FH-PLM on B-chain antibody coupled to Sepharose beads and assaying the supernatant for C-peptide, as well as assaying the eluate from anti-B chain/Sepharose beads for the presence of both B-chain and C-peptide immunoreactivity. In order to perform this experiment, it became necessary to thoroughly characterize the B-chain antiserum and the B-chain antibody coupled to Sepharose beads.

Characterization of B-Chain Antiserum. Fig. 3 demonstrates the radioimmunoassay curves, using the guinea pig anti-bovine insulin B-chain serum. Porcine insulin, desdipeptide proinsulin, desnonapeptide proinsulin, and "split" proinsulin were approximately 2.5 times less immunoreactive than the bovine B-chain on a molar basis. Intact porcine proinsulin was approximately 4.5 times less immunoreactive than the B-chain. The S-sulfonated B-chain-C-peptide fragment prepared by sulfitolysis of desdipeptide proinsulin was approximately 10 times less immunoreactive than the B-chain fragment alone. S-Sulfonated intact porcine proinsulin (straight-chain proinsulin), on the other hand, was approximately 200–250 times less immunoreactive than the B-chain.

Characterization of Anti-B-Chain-Sepharose Beads. Various ¹²⁵I-labeled peptides were tested for their ability to bind to the anti-B-chain-Sepharose beads. The incubations were performed by adding approximately 50,000 cpm of each la-

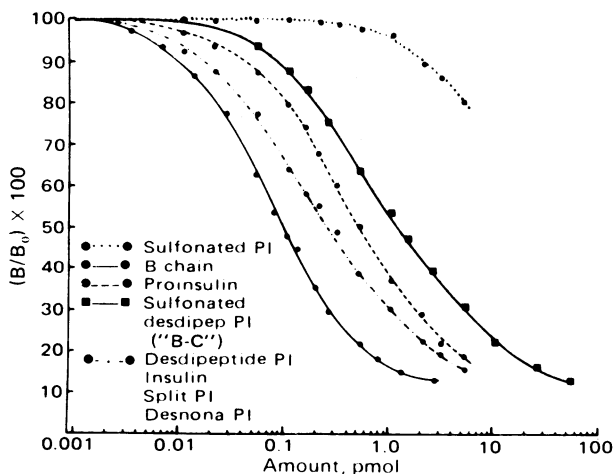


FIG. 3. Characterization of antiserum to B-chain. Immunoassay curves of bovine B-chain, porcine sulfonated proinsulin (PI), "B-C" fragment obtained from sulfonated desdipeptide proinsulin, and proinsulin are shown. Insulin and the intermediate proinsulins showed identical displacement curves. B/B_0 , amount of antibody bound at each dose of antigen.

beled peptide to a 30- μ l gel bed of Sepharose beads in 1 ml of BBSA buffer and mixing on a rotator for 2 hr at room temperature. The mixtures were centrifuged and the supernatants were separated. Table 1 shows that the anti-B-chain-Sepharose beads bind 77% of the insulin label, 66% of the B-chain label, and 33% of the labeled B-chain-C-peptide fragment. Importantly, the 125 I-A-chain and 125 I-C-peptide were not bound to the beads. Incubations with normal nonimmune guinea pig serum bound to Sepharose beads showed virtually no binding of any of the labeled peptides.

Localization of Defect in Familial Hyperproinsulinemia Proinsulin. Highly purified FH-PLM was isolated by using anti-insulin-Sepharose beads. The total isolated material assayed 500 microunits when read on a human insulin standard, or 100 ng of proinsulin on the intact human proinsulin standard. On the assumption that the material represented an intermediate proinsulin, the isolated material would be equivalent to 50 ng in actual mass (see above). Aliquots of 20 ng each were lyophilized and subjected to sulfitolysis in two separate experiments. The sulfonated material was desalted by chromatography on a Bio-Gel P-2 column, lyophilized, and redissolved in 1 ml of BBSA buffer. An aliquot of 740 μ l, theoretically composed (based on M_r 9000) of 4.0 ng of A-chain, 5.62 ng of B-chain, and 4.88 ng of C-peptide, was incubated with a 300- μ l gel bed of anti-B-chain-Sepharose beads in a total volume of 2 ml of BBSA buffer with rotary mixing for 1 hr at room temperature. The

Table 1. Binding of 125 I-labeled peptides to anti-B-chain-Sepharose beads

Peptide	Percentage bound	
	Anti-B-* Sepharose	NGPS:† Sepharose
Porcine insulin	77.1	4.8
Bovine B-chain sulfonate	66.0	4.7
Porcine "B-C" chain sulfonate	33.0	4.2
Porcine A-chain sulfonate	2.7	3.6
Human C-peptide	1.9	2.0
Porcine glucagon	5.0	4.9
Bovine pancreatic polypeptide	2.4	2.4
Synthetic somatostatin	3.4	3.7

* Guinea pig anti B-chain serum.

† Normal guinea pig serum.

Table 2. Localization of defect in familial hyperproinsulinemia proinsulin

	Exp.	B-Chain, ng		C-Peptide, ng	
		Expected	Found	Expected	Found
Supernatant	1	5.62	0	4.88	0
	2	5.62	0	4.88	0
Eluate from					
anti-B-chain	1	5.62	0.40/4.40*	4.88	4.66
Sepharose beads	2	5.62	0.49/5.17*	4.88	4.58

* B-chain equivalent calculated from a porcine S-sulfonated B-chain-C-peptide standard.

mixture was centrifuged and the supernatant was removed for radioimmunoassay. Neither B-chain nor C-peptide was found in the supernatant (Table 2), suggesting that both fragments were removed from the mixture by the anti-B-chain-Sepharose beads. The anti-B-chain-Sepharose beads were then washed with two portions of 2 ml of BBSA buffer, and the washes were discarded. The bound material was eluted from the beads with two portions of 2 ml of glycine-HCl buffer. The eluates were combined and lyophilized, and the glycine was removed by chromatography on a Bio-Gel P-2 column in 3 M acetic acid. Appropriate aliquots of the deglycinated material were separately assayed for the presence of B-chain and C-peptide. Both B-chain and C-peptide were found in the eluate from the anti-B-chain-Sepharose beads (Table 2) indicating that the C-peptide is still attached to the B-chain. C-Peptide recoveries were greater than 90% of the expected amounts calculated from the starting material. B-Chain recoveries were less than 10% of expected when calculated against a B-chain standard. However, the recovery of B-chain equivalent was 79% and 92%, respectively, in the two experiments when calculated from a porcine S-sulfonated B-chain-C-peptide fragment standard. These findings indicate the presence of an intact B-chain-C-peptide fragment in familial hyperproinsulinemia proinsulin.

DISCUSSION

Proinsulin is cleaved into two components, insulin and the connecting peptide or C-peptide, by an enzymatic process within the insulin secretory vesicles in the beta cells. The converting enzymes are thought to be similar to trypsin and carboxypeptidase B, and cleave proinsulin at the sites where the C-peptide is linked to the A and B-chains (7). Trypsin, or a trypsin-like enzyme, cleaves human proinsulin (Fig. 4) at the Arg₆₅-Gly₆₆ residues connecting the C-peptide to the A-chain. The basic residues, Arg-65 and Lys-64 are subsequently cleaved from the carboxyl terminal of the C-peptide by a carboxypeptidase B-like enzyme, giving rise to desdipeptide proinsulin or intermediate proinsulin I. Trypsin additionally cleaves proinsulin at the Arg₃₂-Glu₃₃ residues connecting the B-chain to the C-peptide, resulting in the formation of C-peptide and diarginyl insulin. The two basic arginine residues (Arg-32 and Arg-31) are then removed to yield insulin. In normal subjects, the secretory vesicle contents, consisting of equimolar amounts of insulin and C-peptide and a small amount of intact proinsulin as well as proinsulin intermediates, are released into the circulation. These proinsulin and insulin components can be separated on the basis of their molecular size by gel chromatography of the plasma. The proinsulin peak has been shown to be heterogeneous (8), and consists of a mixture of intact proinsulin and desdipeptide proinsulin. The intermediate forms of proinsulin were originally isolated from crystalline bovine insulin and characterized by Steiner *et al.* (9). Proinsulin intermediate I is a two-chain proinsulin cleaved at the A-chain-C-peptide linkage site and lacks the Lys-Arg sequence, and

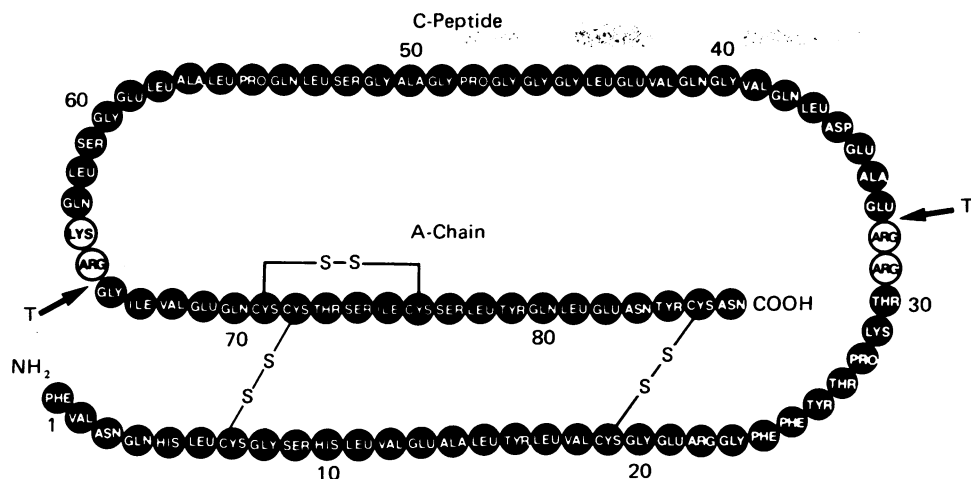


FIG. 4. Amino acid sequence of human proinsulin. Arrows indicate the sites of tryptic cleavage (T). The Arg⁶⁵-Lys⁶⁴ residues and the Arg³¹-Arg³² residues are removed by a carboxypeptidase B-like enzyme to yield insulin and C-peptide. The suspected site of the defect in familial hyperproinsulinemia is at Arg³²-Glu³³.

intermediate II is a two-chain proinsulin cleaved at the B-chain-C-peptide linkage site and lacks the two Arg residues.

In this study, we characterized the circulating PLM in the plasma of patients with familial hyperproinsulinemia (1). The autosomal dominant pattern of inheritance suggested that the defect was likely to be a structural abnormality in the proinsulin molecule, rather than a converting enzyme defect. To distinguish these two possibilities, we studied the conversion of familial hyperproinsulinemia PLM to insulin with trypsin. Incubation of mixtures of FH-PLM and porcine ¹³¹I-proinsulin marker with trypsin showed that the FH-PLM was relatively resistant to tryptic conversion as compared to the porcine proinsulin marker. The conversion of FH-PLM to insulin was both trypsin concentration and time dependent. The ability to completely convert FH-PLM to insulin, albeit at a slower rate than porcine proinsulin, is evidence of a structural defect in FH-PLM. The additional finding of an average 50% molar yield of insulin at various time points in these tryptic conversion studies further suggested that the amount of FH-PLM used in the incubation mixtures (calculated against an intact human proinsulin standard) was overestimated 2-fold in the radioimmunoassay. Because the insulin antibody used in the radioimmunoassay is more immunoreactive with insulin than with intermediate proinsulin (insulin > intermediate proinsulin > intact proinsulin, 5:2.5:1, respectively), the data suggested that FH-PLM may be an intermediate proinsulin.

Supporting data for this inference was derived from studies using two C-peptide antisera differing in their immunoreactivity with intact proinsulin. The two antisera reacted equally with FH-PLM, indicating that FH-PLM is being recognized as a C-peptide rather than as an intact proinsulin—i.e., a two-chain proinsulin intermediate that still has a portion or all of the C-peptide attached to one of the chains of insulin. Presumably, the two antisera are reacting with an exposed C-peptide rather than an intact proinsulin, because the three-dimensional structure of intact proinsulin is responsible for the variation in immunoreactivity with the different C-peptide antisera.

Additional studies of highly purified FH-PLM, isolated with a specific immunoabsorbent column, provided independent confirmation of the two-chain-intermediate nature of FH-PLM, and localized the structural defect to the site linking the B-chain to the C-peptide. Sulfitolysis of FH-PLM to break the interchain disulfide bridges (Fig. 4), followed by immunoadsorption of the B-chain on a specific B-chain antibody coupled to Sepharose beads, demonstrated that the C-peptide immunoreactivity was still associated with the B-chain. Recoveries of C-peptide and B-chain (the latter calculated from a porcine B-chain-C-peptide

fragment standard) were approximately 90% of the expected amount. This confirmed that FH-PLM is a two-chain-intermediate proinsulin with a structural defect at or near the B-chain-C-peptide linkage site.

The structural defect in FH-PLM is presumably a mutation with a single amino acid substitution either at or near the Arg³²-Glu³³ cleavage site connecting the B-chain to the C-peptide. Such a defect is consistent with the benign nature of familial hyperproinsulinemia and the absence of abnormal glucose tolerance in the affected family members. Whereas intact porcine proinsulin has only 18% of the biological activity of insulin on a molar basis, when studied *in vivo*, desdipeptide proinsulin (intermediate I) has 58% of the biological activity of insulin on a molar basis (10). The higher biological activity of intermediate proinsulin suggests that FH-PLM is indeed contributing to the maintenance of normal carbohydrate tolerance in the affected individuals. Additional investigations of familial hyperproinsulinemia may provide insight into the genetics of proinsulin synthesis and the adaptation of the organism to a primary defect in the beta cell secretory product.

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