Alanine Scanning Mutants of Rat Proinsulin I Show Functional Diversity of Anti-Insulin Monoclonal Antibodies

O. Yu Tikhomirov and James W. Thomas

*J Immunol* 2000; 165:3876-3882;

doi: 10.4049/jimmunol.165.7.3876

http://www.jimmunol.org/content/165/7/3876

References

This article cites 34 articles, 13 of which you can access for free at:

http://www.jimmunol.org/content/165/7/3876.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Alanine Scanning Mutants of Rat Proinsulin I Show Functional Diversity of Anti-Insulin Monoclonal Antibodies

O. Yu Tikhomirov and James W. Thomas

In contrast to autoantibodies that are functionally silenced or deleted, IgG Abs that react with autologous insulin routinely follow hormone administration and arise spontaneously in autoimmune (type I) diabetes mellitus. To understand Ab interactions with autologous insulin, rat proinsulin I and 32 alanine substituted analogues were expressed as fusion proteins and used to examine 16 anti-insulin mAb in ELISA. The results identify several amino acid residues that contribute to binding by a large majority (>75%) of mAb, although no single residue is uniformly required for binding by all mAb. Replacements at charged or polar residues on the insulin surface including A4 (Asp), A5 (Gln), A9 (Ser) A12 (Ser), A17 (Gln), A18 (Asn), B13 (Glu), and B21 (Glu) consistently decreased mAb binding. Single alanine substitutions at positions A16 (Leu), A11 (Cys), B8 (Gly), and B15 (Leu) that are predicted to alter the core structure or chain folding vary widely in their impact on Ab binding. mAb that bind insulin preferentially on solid phase (i.e., ELISA) are highly sensitive to replacement of single residues, and substitutions that alter conformation abolish binding. In contrast, high affinity mAb that bind insulin in solution are relatively insensitive to substitutions at single residues, and they maintain binding to all mutants, including those with disrupted conformation. For such high affinity mAb, replacement of long hydrophobic side chains can augment binding, suggesting mAb interactions with insulin include an induced fit. Thus, the ability of insulin to function as a “molten globule” may contribute to the diversity and autoreactivity of the anti-insulin repertoire. The Journal of Immunology, 2000, 165: 3876–3882.

Insulin is a 5808-Da two-chain protein that is produced from a single-chain precursor, proinsulin, in the pancreatic β cells of all mammals. Administration of insulin in the treatment of human diabetes mellitus and immunization of experimental animals routinely results in the production of Abs that bind autologous protein (1–4). Insulin Abs are also found spontaneously following viral infections, drug reactions, and in the autoimmune prodrome of type I (insulin-dependent) diabetes mellitus (5–7). In contrast to autoantibodies that develop as a consequence of immune defects in intracellular signaling (8–10) or apoptosis (11, 12), autoreactivity to insulin may arise in a host with a normal immune system.

Structural studies of mAb that bind autologous insulin show that they are chiefly the products of independent B cell progenitors that have undergone somatic mutation (13–15). Although not clonally restricted, recurrent usage of similar VH, VK, and complementarity-determining region (CDR)3 structures by different mAb suggests that many anti-insulin B cells may recognize similar epitopes on the molecule. However, the number of naturally occurring species variants of insulin that may be used to map specificity is limited, and a clear understanding of the interaction of Abs with insulin is lacking. Furthermore, the detection of insulin binding is recognized to vary depending upon the assay methods. Subsets of mAb and serum Abs are observed to bind preferentially in solid-phase (ELISAs) or in fluid-phase (RIAs) immunoassays (16–19). An important feature of insulin recently recognized in structural studies is that the hormone functions as a “molten globule,” whose configuration is altered by molecular interactions (20, 21). Thus, discordance in binding in different immunoassays may also reflect changes in epitope display related to conformational changes in the insulin globule that are independent of primary structure.

To better understand the structural basis for Ab interaction with autologous insulin, this study takes advantage of the observation that the structure of native insulin is conserved in proinsulin (22, 23). Accordingly, recombinant rat proinsulin I was produced as a purified fusion protein and used to examine immunoreactivity on panel of anti-insulin mAb. For 15 of 16 mAb, binding to the recombinant protein closely parallels that to native insulins and verifies the expected conservation of insulin structure in proinsulin fusion proteins. Extending this approach, individual amino acids from rodent proinsulin were replaced with alanine, and the analogues were used to access how changes in single amino acid side chains impact the interaction with different anti-insulin mAb. The data indicate that most mAb bind insulin through a broad interface that includes several A and B chain residues. mAb that bind insulin preferentially in solid-phase assays are highly sensitive to alterations in conformation as well as to loss of single charged or polar amino acids on the surface of insulin, while mAb that bind insulin in solution are less sensitive to these changes. The key insulin residues predicted to interface with mAb binding sites are amino acids that are highly conserved in phylogeny and thus favor selection of B lymphocytes whose Ig receptors bind autologous insulin. The ability of some mAb to bind conformationally distorted analogues and enhance binding after removal of hydrophobic residues suggests that cooperative interactions between the Ab binding site and the insulin globule are features of high affinity binding.
Materials and Methods

Monoclonal Abs and enzyme immunoassays

Fifteen anti-insulin mAb used in this study were derived from secondary immunization of a single BALB/c mouse immunized with human insulin (15) One mAb (M16) is derived from a different mouse extensively hyperimmunized with insulin conjugated to lactoperoxidase (15). All mAb are from the lgG subclass, and the structures of their expressed VH and VL genes are known. mAb that share recombination events in CDRH3 and CDRL3 are referred to as sibling clones. As described, mAb1, -10, -11, and -13 share the characteristic phenotype of strong binding in ELISA, yet bind poorly to soluble biotinylated or [125I]insulin (24). mAb2, -12, -14, and M16 bind monomeric insulin in solution and on ELISA plates (15, 24). Abs were purified from ammonium sulfate precipitates using protein A-Sepharose (Pharmacia, Uppsala, Sweden), and the linear portion of the binding curve was determined by direct ELISA using goat anti-mouse lgG alkaline phosphatase-conjugated second Ab (Southern Biotechnology Associates, Birmingham, AL). To examine reactivity on different insulins and fusion proteins, Immununol II plates (Dynatech, Alexandria, VA) were coated with 1 μg/ml of the indicated insulin or fusion protein in PBS, pH 8.0. Three different concentrations of mAb from the linear part of the binding curve (OD405, >0.5–1.5) were used to measure binding. Pork proinsulin and human insulin used in these studies were obtained from Dr. Ron Chance (Eli Lilly, Indianapolis, IN). To compare binding to multiple fusion proteins in a uniform fashion, OD data are expressed as the percent binding (rounded to the nearest decile) to that obtained on human insulin or rat proinsulin in the same assay. The methods used for ELISA and RIA were described previously (Refs. 15 and 24 and references therein).

Production of rat proinsulin I fusion proteins

A cDNA clone containing full-length rat preproinsulin 1 sequence was a gift from Dr. Savio L. C. Woo (Howard Hughes Medical Institute, Pittsburgh, PA). Digestion of pBluescript II SK-rINS plasmid with XhoI released a fragment of ~400 bp (25). PCR was used to introduce an NdeI site 5′ in the rat preproinsulin cDNA that maintains the reading frame from Met at position 24 in the pre-peptide. After digestion, the product was then cloned into the NdeI-XhoI site of the pET-14b vector (Novagen, Madison, WI), which carries an N-terminal His-Tag sequence followed by a thrombin site. BL21 competent cells (Novagen) were transformed with pET14b-His Tag rat proinsulin, and individual colonies were obtained. The nucleotide sequence of the clones was confirmed using a 373A DNA sequencer (Applied Biosystems, Foster City, CA). To obtain fusion protein, bacterial cells were grown until an OD600 of ~0.7–0.8 was reached at 37°C in Luria Bertonia broth, and then expression was induced using 1 mM isopropyl β-D-thiogalactoside. Fusion proteins were purified according to recommendations provided by Novagen using 5 M urea and nickel columns (26). Expression of protein in bacterial extracts and quality of purified fusion proteins were examined using 10% PAGE and Coomassie blue staining (27).

Mutagenesis of rat proinsulin I

Alanine substitution of individual amino acids of the insulin sequence was conducted on rat proinsulin cDNA using PCR and the megaprimer approach (28). Briefly, the first round of PCR is conducted using a 5′ primer that contains the mutation of the corresponding residue to alanine and a 3′ primer that contains the XhoI cloning site (5′-CCCTCAGTCAGTGGTCTCCAG). One strand of this product is then used as the 3′ primer in a second round of PCR with a new 5′ primer that contains the NdeI cloning site (5′-CGCCCATATGGCCTTGGATGC). Some mutations close to the 5′ or 3′ end were produced with extended 5′ or 3′ primers containing corresponding mutations in one round of PCR. Mutants containing alanine substitutions were then reintroduced into the NdeI/XhoI site of pET14b, and fusion proteins were obtained as described above. To produce rat proinsulin with amino acids reverted to human insulin sequences, the same approach was used to introduce glutamic acid at A4, glutamine at B3, and serine at B9. Oligonucleotides were synthesized using an Applied Biosystems 392 DNA synthesizer at the Vanderbilt Ingram Cancer Center (Nashville, TN).

Molecular models

Models expressing amino acid side chains of insulin were produced using structural coordinates of human insulin obtained from the Protein Data Base (Brookhaven, NY) and Insight II software (Vanderbilt Ingram Cancer Center).

Results

Production and immunoreactivity of rat proinsulin I as a fusion protein

Structural studies indicate that the conformation of insulin A and B chains derived from the folding of proinsulin is retained in the presence of connecting peptide (c-peptide) (22, 23). Therefore, the single-chain structure of proinsulin provides a useful tool to examine epitopes on multiple proinsulin analogues without laborious

FIGURE 1. Production of a rat proinsulin fusion protein. A, Coomassie blue stain of a polyacrylamide gel showing the induction of cloned rat proinsulin 1 as a His-Tag fusion protein: lane 1, Molecular weight markers; lane 2, induced vector control; lane 3, uninduced vector control; lane 4, induced rat proinsulin fusion protein; lane 5, uninduced rat proinsulin clone; lane 6, purified pork proinsulin. B, Coomassie blue stain of affinity-purified His-Tag proinsulin protein at 10 and 1 μg/ml. C, Western blot on a series of rat proinsulin fusion proteins using a pool of anti-insulin mAb (lanes 4–12): lane 1, pork proinsulin control; lanes 2 and 3, loaded with eluates from control cultures.

FIGURE 2. Representative ELISA demonstrating binding of mAb2 on human insulin (Hins), pork proinsulin (Pk-Proins), and recombinant rat proinsulin fusion protein. Data are OD405 for the indicated concentration of purified mAb.
recombination of individual chains. Rat proinsulin I cDNA (identical with mouse insulin I sequences) was cloned into the pET14b vector and expressed as a fusion protein with a His-Tag peptide at its amino-terminal end (see *Materials and Methods*). After induction, the fusion protein was purified on nickel columns and examined by PAGE and immunoblotting using a pool of anti-insulin mAb. The data in Fig. 1 show the induction of rat proinsulin I fusion protein (lane 4) of the expected m.w. compared with pork proinsulin (lane 6). Most contaminating material is removed by affinity purification on nickel columns (lanes 1 (10 mg), lane 2 (1 mg)). Shown in Fig. 1C is an immunoblot on recombinant rat proinsulin fusion proteins (lanes 4–12) and pork proinsulin (lane 1) using a pool of 10 anti-insulin mAb; bacterial extracts from vector controls are not immunoreactive (lanes 2 and 3). These findings are consistent with the conserved structure of insulin in proinsulin and confirm that recombinant rat proinsulin fusion protein may serve as a target for characterizing mAb recognition of insulin.

**Conversion of rat proinsulin to a human-rat proinsulin chimeric protein**

mAbs from the secondary response of BALB/c mice to human insulin were used to further examine the immunoreactivity of our recombinant rat proinsulin fusion protein. Previous studies show that 15 of 16 mAb derived from the secondary response to human insulin bind rodent insulins with 60–100% of their immunoreactivity on human insulin (15). These mAb also had comparable binding to pork proinsulin, indicating that proinsulin retains most of the immunoreactivity of native insulin, and the presence of c-peptide does not interfere with Ab binding. Accordingly, the immunoreactivity of these 16 mAb was tested on our rat proinsulin fusion protein. Shown in Fig. 2 is a representative binding curve of mAb2 to human insulin, pork proinsulin, and rat proinsulin I fusion protein in the ELISA. The data are summarized in Table I, where the immunoreactivity to humanized rat proinsulin is presented as a percentage.

**Table I. Immunoreactivity of rat proinsulin fusion protein**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Pork Proinsulin</th>
<th>Rat Recombinant Proinsulin</th>
<th>A4, B3, B9 &quot;Humanized&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M16</td>
<td>90</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>130</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>80</td>
<td>100</td>
</tr>
</tbody>
</table>

aData are presented as percent binding compared to human insulin.

bResidues of rat proinsulin replaced with amino acids in human insulin.

Because the rodent insulins I and II differ at three residues (A4, B3, and B9) from the amino acids in human insulin, we wished to determine how these species-specific differences influence mAb recognition. Accordingly, these three residues in the rat proinsulin I fusion protein were converted to the human amino acid sequence using the megaprimer approach (see *Materials and Methods*). The results are summarized in Table I, column 3, where the immunoreactivity between human and rodent insulins is also present in a recombinant rat proinsulin fusion protein.

**FIGURE 3.** mAb that bind insulin in an avidity-dependent manner (ELISA) are highly sensitive to alanine substitutions at single residues. *Top panel,* Graphical representation of anti-insulin mAb1, -10, -11, and -13 binding to rat proinsulin I fusion proteins containing an alanine substitution at the indicated A chain amino acid. *Lower panel,* Summary of binding to alanine-substituted B chain residues for the same mAb. The single-letter amino acid codes above the line graph correspond to the residual number on the x-axis that is substituted with alanine. Data are presented as the percent binding relative to OD at 0.45 on rat proinsulin.
of binding to human insulin. The binding of 12 mAb to the fusion protein did not change or changed <20% when the human residues were inserted. For four mAb, the binding increased 20–40% when rat proinsulin was substituted with the corresponding sequence A4 and B3 (Lys→Gln), and B9 (Pro→Ser) insulin residues. One mAb from the secondary response to human insulin, mAb15, does not bind commercial preparations of rodent insulin (15). As anticipated, mAb15 does not bind the rat proinsulin fusion protein, and a 30% increase in binding is detected when the fusion protein contains human amino acids at residues A4, B3, and B9. The failure to reconstitute >50% of the binding to human insulin in this mAb may reflect the unique nature of the epitope recognized, which is also suggested by its weak reactivity on pork proinsulin (Table I) (15). Thus, the presence of c-peptide may limit optimal interaction of this mAb with native insulin. For most anti-insulin mAb, the data indicate that a recombinant proinsulin fusion protein can provide information on the interaction of anti-insulin mAb with autologous insulin.

**Binding of avidity-dependent anti-insulin mAb to alanine-substituted rat proinsulin analogues**

Insulin Abs and mAb are often recognized to differ in their activity in immunoassays (16, 19, 29, 30). Some mAb prefer to bind insulin on surfaces (e.g., ELISA) potentially due to a requirement for multivalent interactions and/or differences in epitope display. mAb1, -10, -11, and -13 from the secondary response to human insulin show this avidity-dependent phenotype. These mAb bind to insulin strongly in ELISA or to insulin complexes in solution, but do not bind soluble insulin (24). To determine how such mAb recognize insulin, alanine scanning mutants of our rat proinsulin I fusion protein were examined for mAb binding. In this analysis eight amino acids in the B chain terminus were not mutagenized, because these mAb bind desoctapeptide insulin from which the last eight amino acids of the B chain are removed (15). The findings are summarized in Fig. 3 and in Tables II and III, where the relative binding to proinsulin analogues is expressed as a percentage of binding to rat proinsulin in ELISA. The data show that avidity-dependent anti-insulin mAb are highly sensitive to changes in insulin structure. For different mAb, alanine replacement results in >50% loss of binding when any one of 8–11 A chain residues or 7–14 B chain residues are substituted. Alanine replacement at five residues from the insulin A chain have a major impact (i.e., ≥90% decrease) or abolish binding, these are A4 (Glu), A11(Cys), A12(Ser), A16 (Leu), and A17 (Glu). Similar dramatic decreases in binding are observed when side chains are substituted with alanine at B chain residues, these include B13 (Glu), B15 (Leu), and B21 (Glu). Substitution at B8 (Gly), which is important for chain folding, is also observed to result in a large decrease in binding. The data indicate that anti-insulin mAb, which bind in an avidity (ELISA)-dependent manner, are highly sensitive to single amino acid changes that alter either conformation or surface charge.

**Binding of high affinity anti-insulin mAb to alanine substituted rat proinsulin analogues**

mAb2, -12, -14, and M16 are representative of high affinity mAb that effectively bind monomeric insulin in solution. In ELISA, the binding of these mAb is inhibited by low concentrations of soluble insulin (15, 24). Results of direct binding in ELISA using these mAb and alanine scanning mutants of proinsulin are shown in Fig. 4 and Tables II and III. No single amino acid replacement in either A or B chain has a large (>90% decrease) impact on binding to proinsulin analogues. This is true for both amino acids predicted to

---

**Table II. Alanine scanning mutants of rat proinsulin: immune reactivity of insulin A chain residues**

<table>
<thead>
<tr>
<th>Amino Acid and Residue No.</th>
<th>G</th>
<th>I</th>
<th>V</th>
<th>E</th>
<th>Q</th>
<th>S</th>
<th>I</th>
<th>C</th>
<th>S</th>
<th>Y</th>
<th>Q</th>
<th>L</th>
<th>Q</th>
<th>N</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mAb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>0.5</td>
<td>40</td>
<td>40</td>
<td>20</td>
<td>5</td>
<td>10</td>
<td>60</td>
<td>50</td>
<td>0.5</td>
<td>7</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>60</td>
<td>100</td>
<td>60</td>
<td>30</td>
<td>60</td>
<td>75</td>
<td>40</td>
<td>50</td>
<td>70</td>
<td>50</td>
<td>25</td>
<td>40</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>M16</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>30</td>
<td>50</td>
<td>25</td>
<td>80</td>
<td>30</td>
<td>30</td>
<td>100</td>
<td>70</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>60</td>
<td>100</td>
<td>0.5</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>0.5</td>
<td>10</td>
<td>90</td>
<td>60</td>
<td>0.5</td>
<td>3</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>10</td>
<td>50</td>
<td>0.5</td>
<td>70</td>
<td>30</td>
<td>15</td>
<td>0.5</td>
<td>5</td>
<td>90</td>
<td>30</td>
<td>0.5</td>
<td>3</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>100</td>
<td>140</td>
<td>100</td>
<td>100</td>
<td>60</td>
<td>170</td>
<td>70</td>
<td>80</td>
<td>130</td>
<td>120</td>
<td>70</td>
<td>100</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td>0.5</td>
<td>50</td>
<td>20</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>70</td>
<td>10</td>
<td>0.5</td>
<td>5</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>100</td>
<td>250</td>
<td>70</td>
<td>70</td>
<td>75</td>
<td>300</td>
<td>80</td>
<td>130</td>
<td>100</td>
<td>80</td>
<td>20</td>
<td>80</td>
<td>60</td>
<td>130</td>
</tr>
</tbody>
</table>

* Data are percent binding in ELISA compared to OD405 on rat proinsulin.

---

**Table III. Alanine scanning mutants of rat proinsulin: immunoactivity of insulin B chain residues**

<table>
<thead>
<tr>
<th>Amino Acid and Residue No.</th>
<th>F</th>
<th>N</th>
<th>Q</th>
<th>H</th>
<th>G</th>
<th>P</th>
<th>H</th>
<th>V</th>
<th>E</th>
<th>L</th>
<th>Y</th>
<th>L</th>
<th>V</th>
<th>G</th>
<th>E</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mAb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>100</td>
<td>60</td>
<td>60</td>
<td>10</td>
<td>40</td>
<td>50</td>
<td>100</td>
<td>3</td>
<td>7</td>
<td>100</td>
<td>70</td>
<td>20</td>
<td>25</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>100</td>
<td>70</td>
<td>60</td>
<td>25</td>
<td>60</td>
<td>90</td>
<td>80</td>
<td>35</td>
<td>50</td>
<td>85</td>
<td>90</td>
<td>60</td>
<td>90</td>
<td>50</td>
<td>65</td>
</tr>
<tr>
<td>M16</td>
<td>80</td>
<td>100</td>
<td>70</td>
<td>50</td>
<td>30</td>
<td>100</td>
<td>110</td>
<td>80</td>
<td>40</td>
<td>45</td>
<td>100</td>
<td>45</td>
<td>50</td>
<td>90</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>90</td>
<td>60</td>
<td>60</td>
<td>15</td>
<td>60</td>
<td>35</td>
<td>60</td>
<td>7</td>
<td>10</td>
<td>80</td>
<td>60</td>
<td>35</td>
<td>45</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>75</td>
<td>50</td>
<td>40</td>
<td>5</td>
<td>15</td>
<td>7</td>
<td>50</td>
<td>1</td>
<td>3</td>
<td>50</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>140</td>
<td>100</td>
<td>90</td>
<td>50</td>
<td>120</td>
<td>130</td>
<td>100</td>
<td>60</td>
<td>100</td>
<td>120</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>30</td>
<td>50</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>40</td>
<td>0.5</td>
<td>2</td>
<td>50</td>
<td>40</td>
<td>25</td>
<td>20</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>60</td>
<td>170</td>
<td>100</td>
<td>100</td>
<td>60</td>
<td>140</td>
<td>200</td>
<td>140</td>
<td>50</td>
<td>120</td>
<td>150</td>
<td>240</td>
<td>100</td>
<td>180</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Data are percent binding in ELISA compared to OD405 on rat proinsulin.
disrupt conformation as well as for charged or polar amino acids that are present on the molecular surface. Alanine substitution at A16 (Leu) reduces the binding by 30–90%, and substitution at residue A11 (Cys) reduces binding by 20–70%. For mAb2 and M16 loss of charged residues at A5 (Gln) and A4 (Asp), respectively, decreases binding by 70%. Interestingly, for mAb12 and -14, alanine replacement at A4 (Val), A10 (Ileu), or A19 (Tyr) augments binding, possibly reflecting the removal of hydrophobic side chains that leads to an improved fit for these mAb. Alanine substitution in some B chain residues had a moderate impact on binding, as seen at residue B8 (Gly) and B13 (Glu) for all four mAb (75–40% decrease). The activities of mAb2 and mAb M16 are decreased when residues B15 (Leu) and B21 (Glu) are replaced, but changes at these residues have little impact on the binding of mAb12 and -14. Replacement of several residues in the insulin B chain with alanine results in a dramatic increase (>50%) in binding by mAb14; these residues include B3 (Gln), B10 (His), B13 (Glu), B16 (Glu), B17 (Leu), and B20 (Glu). Interestingly, these changes were not seen with mAb10 when these same residues were converted to alanines. mAb10 is structurally very similar to mAb14 and on the basis of CDRH3 structure is predicted to

![Figure 4](image1.png) mAb that bind insulin in solution are relatively insensitive to alanine substitutions at single residues. Upper panel, Graphical representation of anti-insulin mAb2, -12, -14, and M16 binding to rat proinsulin I fusion protein containing an alanine substitution at the indicated A chain amino acid. B (lower panel) summarizes binding to alanine substituted B chain residues for the same mAb. The single-letter amino acid codes above the line graph correspond to the residual number on the x-axis that is substituted with alanine. Data are presented as the percent binding relative to OD405 on rat proinsulin.

![Figure 5](image2.png) Top, Space-filling model showing the positions of charged amino acid side chains at residues A4 (Asp), A12 (Ser), A17 (Gln), B13 (Gln), and B21 (Gln) that are predicted to provide contacts for most insulin Abs. Bottom, Ribbon model of the insulin backbone (A chain in black) viewed down the a-helix of the B chain (gray). Cystines and side chains of hydrophobic amino acids including A16 (Leu) and B16 (Leu) that form a hydrophobic core in the molecule are shown. The B chain-terminal six amino acids have been removed to expose the core structures.
be derived from the same parent clone. Thus, differences in binding to analogues may reflect the structural evolution of anti-insulin V regions in the course of the immune response. Overall, the binding of these high affinity anti-insulin mAb is qualitatively less sensitive to individual alanine substitutions than is binding of mAb that bind insulin in an avidity-dependent (solid phase) fashion. However, substitutions at residues responsible for maintaining the hydrophobic core (e.g., A11, A16, and B15) and chain folding (e.g., B8) as well as charged and polar amino acids at multiple surface residues consistently reduce binding by both types of mAb.

**Discussion**

In this study a recombinant rat proinsulin I fusion protein and alanine scanning mutants were used to characterize the immune interactions between mouse anti-insulin mAb and autologous rodent insulin. As expected, 15 of 16 mAb from BALB/c mice immunized with human insulin show binding to a recombinant proinsulin-containing rodent insulin sequences. These findings are consistent with structural studies that show that the conformation of insulin is retained in proinsulin (22, 23) and verify the usefulness of the technique. While the approach may not be applicable to all anti-insulin mAb, the results with 15 of 16 mAb indicate that recombinant rat proinsulin is a useful Ag for examining a large majority of anti-insulin mAb.

To understand interactions with autologous insulin, alanine scanning mutants of our rodent proinsulin fusion protein are used to identify the contributions of individual residues to binding in ELISA. Because direct binding in ELISA is insensitive to small and moderate differences in affinity (31, 32), large changes in binding observed after substitution of alanine for a single amino acid probably identify key sites that interact with Ab. In addition, one or more mAb maintains 100% reactivity with each analogue; thus, changes in binding are not easily explained by loss of insulin adhering to plates or transmission of broad structural changes across the molecule. We find that the binding of an individual mAb may be sensitive to substitution at as many as 14 residues, a number similar to contacts observed for other protein Ags (33). None of 31 residues examined was ignored by all mAb, but positions A1, B1, and B3 were the least reactive. Although no single amino acid substitution proved key for the binding of all mAb, five charged or polar residues on the solvent surface of insulin are repetitively identified as contacts for 75% of mAb. These are B13 (Glu), A17 (Gln), A4 (Asp), B21 (Glu), and A12 (Ser; Fig. 5, top). Substitution of residues A9 (Ser) and A18 (Asn) do not change binding dramatically, but are also found to influence most mAb. Because no two mAb show precisely the same sensitivity to alanine replacements, the overall fine specificity of insulin binding appears to remain diverse even though this set of mAb uses structurally similar V\textsubscript{H}, V\textsubscript{K}558 and V\textsubscript{K}19 genes (15). In the future these mAb and proinsulin analogues will be useful tools for determining whether similar diversity is present in spontaneous autoantibodies in the prodrome of IDDM.

These data differ from a recent study of insulin analogues binding to the hormone receptor where substitution of residues A19 (Tyr) and B13 (Glu) are most critical for interactions with the hormone receptor (34). As in studies of the hormone receptor, replacement of amino acids that contribute to the hydrophobic core and to proper folding of the insulin molecule also alter Ab interactions. These residues include A16 (Leu), A11 (Cys), B8 (Gly), and B15 (Leu) (22). The positions of the hydrophobic core residues relative to the individual A and B chains are shown in Fig. 5 (bottom).

Two general patterns of reactivity with the alanine-substituted analogues are observed to correspond to previously recognized properties of insulin binding Abs. Avidity (i.e., ELISA)-dependent mAb require a precise display of charged amino acid side chains combined with an intact conformation of insulin. This binding appears to depend upon a composite of multiple weak interactions, because changing of a single residue at any one of multiple sites results in >50% loss of mAb binding. Because these are IgG Abs captured in a secondary immune response, they probably reflect selection and differentiation of anti-insulin B cells. The display of insulin on a cell surface (possibly follicular dendritic cells) may favor these interactions. A different degree of sensitivity to alanine substitution is observed in high affinity mAb (e.g., mAb2, -12, -14, and M16) that bind monomeric insulin in solution. In the autoimmune prodomes of IDDM, such Abs are more predictive of the pathological process (16, 29, 35). For these mAb, replacement of individual amino acids with alanine has only a modest qualitative impact on binding, although they may share interaction sites with mAb that only bind in ELISA (avidity dependent). For mAb12 and -14, replacement of several long chain hydrophobic amino acids with alanine results in enhanced binding. This is dramatically illustrated in mAb14, where alanine replacements for valine at A3 or for isoleucine at A10 more than doubles binding. The binding of its sibling clone, mAb10, that differs in four amino acids (one in V\textsubscript{H} and three in CDRs) does not demonstrate enhanced binding with any alanine substitution. These observations favor the postulate that some mAb may acquire an induced fit for the insulin molecule as a consequence of selection and mutation of low affinity precursors whose original interactions were avidity dependent. To explore the relationship between binding in solution and avidity, we have used proinsulin mutants to inhibit the binding of mAb2, -12, and -16 to human insulin. Some substitutions, such as A16 (Leu), which effects conformation, result in loss of binding in solution, but others, such as B8 (Gly), do not (work in progress). We are currently extending these studies to determine the relationship of avidity, affinity, and conformation changes to the differential detection of insulin binding in solution and in solid-phase assays.

The recently recognized capacity of insulin to function as a molten globule may contribute to these structural interactions. The observation that a functionally inactive insulin molecule has the same structure as a nonfunctional molecule led to the proposal that changes in insulin accompany its interaction with the hormone receptor (20, 21). Structural movements in the insulin molecule may also influence mAb binding or the interaction of the B cell receptor with insulin. For example, display of insulin epitopes on a solid phase may stabilize interaction sites for some mAb, while other mAb are competent to mold insulin into a favorable configuration for binding in solution. The ability of mAb2, -12, -14, and M16 to bind insulins with disrupted disulfide bonds (e.g., A11) supports this postulate. The failure of hyperimmunization with heterologous insulin to enrich for mAb that recognize foreign epitopes may reflect a selective advantage for interactions that re-model the interface between the molten globule and the Ab binding site. The contact sites that are optimal for Ab or B cell receptor to form such cooperative interactions are highly conserved in vertebrate evolution and lead to the selection of autoactivity as a routine component of anti-insulin repertoires. In the future a more precise analytical approach will be required to refine our understanding of how structural features of insulin may shape immune repertoires.

**Acknowledgments**

We thank Drs. Larry Moss and Savio Woo for providing the rat proinsulin I gene, and Dr. Marcia Newcomer for assistance with molecular models. We also thank Elaine Beeler for assistance with preparation of the manuscript.
References


