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Epitope analysis of human insulin and intact proinsulin

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Residues belonging to epitopes on human insulin that were recognized by a panel of three monoclonal antibodies were located using mutated insulins and insulins from a number of different animal species. Epitopes on human proinsulin recognized by two monoclonal antibodies were also identified using partially processed proinsulin species. Epitopes were located on the C–A and B–C junctions of proinsulin and on the N-termini of the A- and B-chains and the central region of the B-chain of human insulin. Antibodies that bound proinsulin were found to induce conformational changes in the prohormone. The presence of a well-defined interaction between the C-peptide portion and the N-terminus of the A-chain of the insulin moiety of intact proinsulin has also been demonstrated. The relevance of these studies to the development of two-site assays for the measurement of partially processed proinsulin species in human sera is also discussed.
Key words: antibodies/epitopes/immunoassay/insulin/proinsulin

Introduction

The problem of measuring insulin specifically in human serum is compounded by the presence of both intact proinsulin and partially processed proinsulins (Gray *et al.*, 1984). Assays capable of specifically measuring the wide range of insulin-like molecules in plasma have been achieved by the use of a panel of monoclonal antibodies (mAbs) raised against these molecules (Sobey *et al.*, 1989). Unfortunately these assays are not capable of differentiating absolutely between intact proinsulin and the two split proinsulins and were not sensitive enough to detect fasting levels of 65–66 split proinsulin in human sera. In an attempt to understand and overcome the problems of assay specificity we have determined the epitopes on insulin that are recognized by these mAbs. Accurate information about the sites at which mAbs bind to insulin and proinsulin-like molecules also provides information about the structure of proinsulin and its derivatives. Such information is of interest since little X-ray crystallographic and NMR data, except for that of Weiss *et al.* (1990), exists for these molecules.

The ability of mAbs to bind both insulins of known sequence from different species and mutated insulins containing discrete amino acid substitutions (Brange *et al.*, 1988) was studied and competition assays between mAbs for proinsulin epitopes were also carried out. Two mAbs, ANT1 and A6, were also studied for their ability to differentiate between proinsulin and its partially processed forms and the data gained from such experiments yielded some information concerning the structure of these molecules.

Materials and methods

Reagents

Radiolabelled insulin, intact proinsulin and des-31–32 proinsulin were obtained from Eli Lilly (Indianapolis, IN). Mutant insulins and rat and human (Actrapid) insulin were obtained from Novo Industri A/S (Bagsværd, Denmark). Human C-peptide, bovine, equine and ovine insulins were obtained from Sigma (Poole, Dorset, UK). Porcine insulin (Velosulin) was obtained from Nordisk-Wellcome (Gentofte, Denmark). Chemically modified insulins and intact, 65–66 split, des-64–65-, 32–33 split and des-31–32 human proinsulins were kindly donated by Dr R.Chance of Lilly Research Laboratories (Indianapolis, IN). Microtitre plates (Nunc-immuno module, maxisorp F16) were obtained from Nunc (Roskilde, Denmark).

Antibodies

The mAbs A6 and 3B1 were prepared from mice immunized with human proinsulin (Gray *et al.*, 1987; Sobey *et al.*, 1989) and the mAb 14B was prepared from mice immunized with human insulin (Sobey *et al.*, 1989). The mAbs 1E2 and ANT1 were the products of separate hybridoma cell clones that originated from the same fusion experiment. This fusion involved spleen cells taken from a mouse that had been immunized with three different immunogens, i.e. human des-31–32 proinsulin, intact proinsulin and insulin, each immunogen having been injected separately using the method of Sobey *et al.* (1989). Scatchard analysis was used to determine the binding affinities of the mAbs using the technique of Soos and Siddle (1982). The dissociation constants for the mAbs were ANT1, 1.73 nM for des-31–32 proinsulin and 1.61 nM for intact proinsulin; A6, 0.25 nM for intact proinsulin; 1E2, 0.79 nM for insulin and 1.12 nM for intact proinsulin; 3B1, 0.12 nM for intact proinsulin and 0.11 nM for insulin; 14B, 9.0 nM for insulin. Thus, the binding affinities of the antibodies for insulin covered a range spanning almost two orders of magnitude.

Displacement assays

To determine the insulin epitopes recognized by the mAbs 3B1, 1E2 and 14B, the antibodies were incubated with iodinated human insulin in the presence of non-labelled insulins from different species, i.e. porcine, bovine, ovine, rodent and equine and mutated insulins. The amino acid sequences of some of these insulins are shown in Table I. The assay protocol was as follows: ^{125}I-labelled human insulin (sp. act. 374 μCi/μg) was diluted in 50 mM sodium barbitone buffer (50 mM sodium barbitone, 90 mM NaCl, 1.5 mM NaN_3, 14 mM HCl and 0.5% BSA, pH 8.0) and 50 μl aliquots were added to Eppendorf tubes (~6000 c.p.m./tube). Non-labelled insulin of known sequence was added at increasing concentrations, 50 μl/tube in 50 mM sodium barbitone buffer followed by a 100 μl aliquot of the particular mAb under study. The mAb was used at a concentration that gave 30–50% of maximal binding of the ^{125}I-labelled insulin. Incubation was carried out at 4°C for 24 h followed by addition of 100 μl/tube of sheep antimouse immunoglobulins coupled to aminocellulose (Hales and

Table I. Insulins used for epitope mapping studies

Name	Residues that differ from human insulin
A0F,DesB30	Phe added to A1 and B30 deleted
A4Q,B21Q	A4 Gln (Glu), B21 Gln (Glu)
A8H	A8 His (Thr)
A21G	A21 Gly (Asn)
B1E,B27E	B1 Glu (Phe), B27 Glu (Thr)
B3-desamido	B3-desamido Arg (Arg)
B9D,B27E	B9 Asp (Ser), B27 Glu (Thr)
B10T	B10 Thr (His)
B13Q	B13 Gln (Glu)
B16Q	B16 Gln (Tyr)
B27E	B27 Glu (Thr)
DesB26–B30	deletion of 5 C-terminal residues of insulin B-chain
B31,B32 insulin	two Arg residues added to C-terminus of insulin B-chain
Bovine	A8 Ala (Thr), A10 Val (Ile), B30 Ala (Thr)
Equine	A9 Gly (Ser), B30 Ala (Thr)
Ovine	A8 Ala (Thr), A9 Gly (Ser), A10 Val (Ile), B30 Ala (Thr)
Porcine	B30 Ala (Thr)

Residues in brackets are those found in human insulin.

Woodhead, 1980). The incubation period was 2 h at room temperature and was followed by addition of 1 ml/tube of 25 mM sodium barbitone buffer containing 0.5% Tween-20 and centrifugation at 4000 *g* for 5 min. The supernatants were aspirated and the wash procedure repeated once more. Pellets were counted for 1 min in a Nuclear Enterprises 1600 Gamma Counter.

Similar experiments were carried out using mAbs ANT1 and A6 in the presence of either [^{125}I]des-31–32- or intact pro-insulin and non-labelled human C-peptide, insulin, diarginyl (B31, B32) insulin, des-64–65-, 65–66 split, des-31–32-, 32–33 split and intact proinsulin. Non-human proinsulins were not used in these studies since other animal proinsulins have very different C-peptide amino acid sequences and, therefore, would not yield any useful information in terms of epitopes recognized by our mAbs on the prohormone.

Monoclonal antibody competition assays

These experiments were carried out to assess the ability of one mAb to bind antigen in the presence of a second mAb. The first antibody was absorbed onto a solid phase (wells of a microtitre plate) by first diluting to a concentration of 50 μg/ml in Tris buffer (10 mM Tris, 0.1 M NaCl and 1.5 mM NaN_3, pH 8.5) and adding 200 μl to each well. The plate was then left at 4°C for 24 h after which the supernatants were aspirated from the treated wells and 350 μl of Tris buffer containing 0.05% Tween-20 was added to each and then poured off. This wash procedure was repeated twice more. To each well was then added 250 μl of a 1% BSA solution made up in the Tris buffer and this was left at room temperature for 2–3 h. The wash procedure was then carried out twice using 25 mM sodium barbitone buffer and once with 25 mM sodium barbitone buffer containing 0.5% Tween-20. To each well was added 50 μl of the second mAb (stock solution of 7–10 mg/ml) diluted 500–500 000-fold in 50 mM sodium barbitone buffer. [^{125}I]Proinsulin was added (50 μl/well which gives ~20 000 c.p.m./well at a sp. act. of 15 μCi/μg) and incubated at 4°C for 24 h. The supernatants were then aspirated and each well was washed once with 25 mM sodium barbitone buffer and counted for 1 min in a Nuclear Enterprises 1600 Gamma Counter.

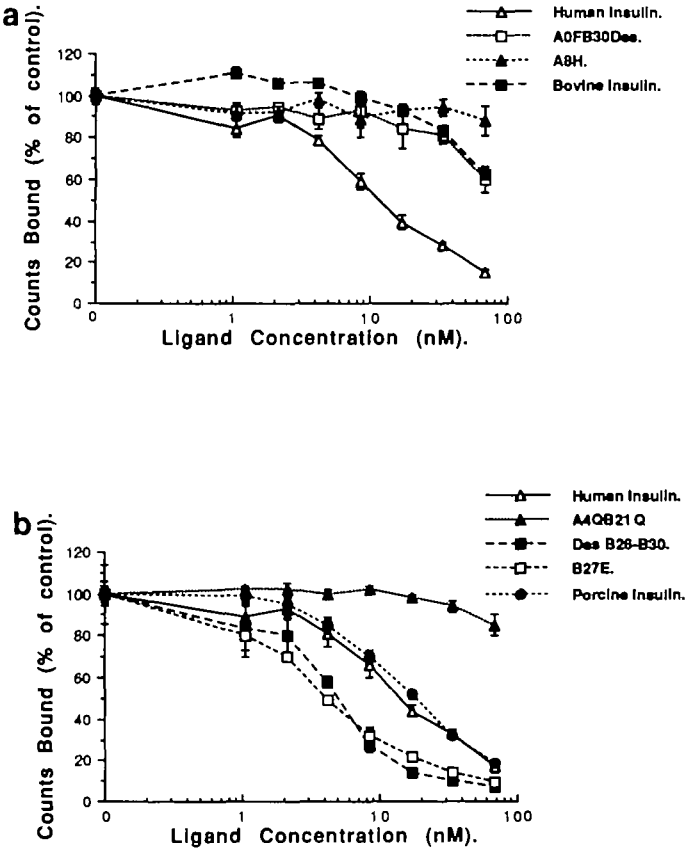


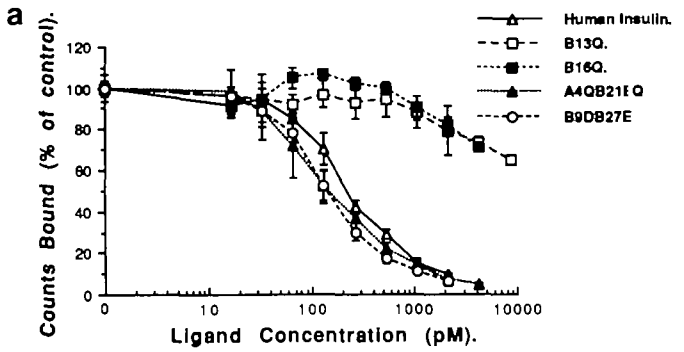
Fig. 1. (a) and (b) Insulin displacement curves for mAb 14B. The antibody was incubated with [^{125}I]human insulin in the presence of increasing concentrations of unlabelled mutant or animal insulins. Each point on all the curves represents the mean of 4–12 observations ± SEM.

Results

Displacement assays

Displacement assays with 14B (Figure 1a and b) showed that bovine insulin was less effective at displacing the iodinated human hormone compared to non-labelled human insulin (Figure 1a) whilst porcine insulin was as equally effective as the human hormone (Figure 1b). Equine and ovine insulin were also tested and gave data very similar to that for bovine insulin (data not shown). Figure 1(a) also shows that the mutant insulin A0F,DesB30 produced displacement of labelled insulin from 14B comparable to that of bovine insulin. The insulin A8H however was ineffective in displacing the labelled human insulin from the mAb, i.e. over the concentration range at which the mutant insulin was used it was unable to bring down binding of [^{125}I]human insulin to 14B to a level below 80% of that of the control binding. The control binding is defined as the level of binding of the [^{125}I]insulin to the mAb under study that occurs in the absence of any competing insulin and is arbitrarily set at 100%. In Figure 1(b) the mutant insulin A4Q,B21Q was also ineffective at displacing human insulin whilst both the B27E and des-(B26–B30)-insulins were better than the human insulin. A mutant insulin with a change at residue A21 was equally effective as human insulin in displacing the labelled hormone from the antibody (data not shown).

With 3B1 (Figure 2a and b), the mutant insulins B16Q and B13Q were much less effective at displacing the labelled human insulin from the mAb compared to human insulin (Figure 2a), whilst the insulin B10T was four times less effective compared



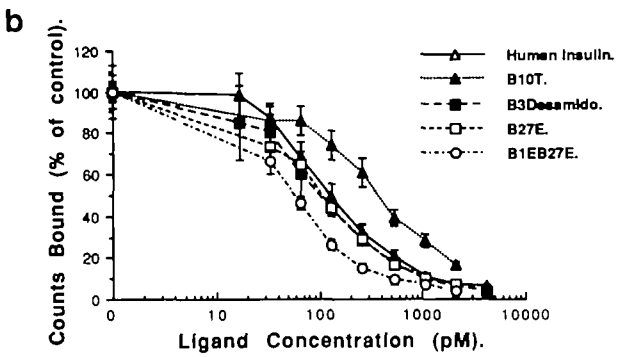
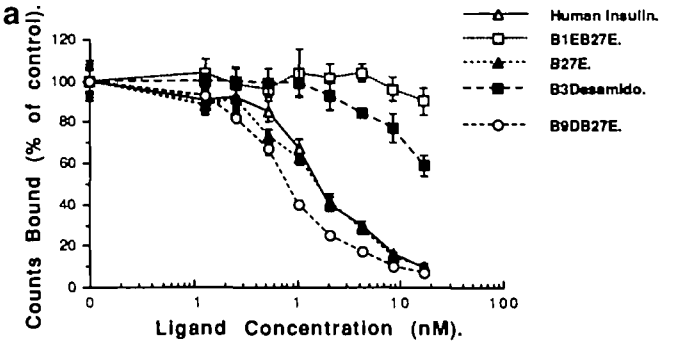


Fig. 2. (a) and (b) Insulin displacement curves for mAb 3B1. The antibody was incubated with [^{125}I]human insulin in the presence of increasing concentrations of unlabelled mutant. Each point on all the curves represents the mean of 4–12 observations ± SEM.



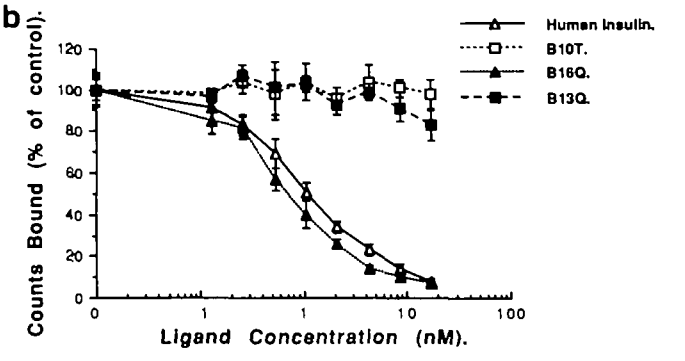


Fig. 3. (a) and (b) Insulin displacement curves for mAb 1E2. The antibody was incubated with [^{125}I]human insulin in the presence of increasing concentrations of unlabelled mutant. Each point on all the curves represents the mean of 4–12 observations ± SEM.

to human insulin (Figure 2b). The insulins A4Q,B21Q, B9D,B27E (Figure 2a), B27E and B3-desamidoinsulin (Figure 2b) were as potent as the unaltered human hormone. The B1E,B27E mutant insulin was found to be more effective than human insulin at displacing the radiolabelled species (Figure 2b).

As shown in Figure 3(a), the mAb 1E2 was ineffective in binding insulin B1E,B27E and only slightly better at binding B3-desamidoinsulin. It bound insulin B27E to the same extent and mutant insulin B9D,B27E more strongly compared to normal human insulin. Figure 3(b) demonstrates that 1E2 was ineffective in binding insulin with mutations at residues B10 and B13 but was able to bind insulin with an altered residue at position B16.

The results of displacement assays using the mAb ANT1 are shown in Figure 4(a and b). Figure 4(a) shows that neither C-peptide or insulin was capable of displacing radiolabelled des-31–32 proinsulin from the antibody. Further studies showed that ANT1 was incapable of binding [^{125}I]C-peptide and that C-peptide was unable to displace [^{125}I]proinsulin from the mAb even at a concentration (9 μM) that was 260 000-fold higher than that of the iodinated ligand (data not shown). Figure 4(b) shows that the des-31–32- and 32–33 split proinsulins were equally potent at displacing labelled proinsulin whilst the des-64–65– and 65–66 split proinsulins were ten times less potent with the split molecule being slightly better than the des-64–65 at displacing the labelled ligand. Figure 4(c) shows that 65–66 split and intact proinsulin were equipotent for displacement of radiolabelled proinsulin from mAb A6 whilst 32–33 split proinsulin was 30 times less potent. The des-31–32 proinsulin was slightly less effective than the split molecule at displacing the labelled ligand. Insulin was found to be incapable of displacing the labelled prohormone from A6 whilst a modified insulin containing two extra Arg residues at positions 31 and 32 of the insulin B-chain was capable of displacing labelled proinsulin. Further studies also showed that C-peptide was not bound by A6 (data not shown).

Competition assays

Figure 5(a) shows that the mAb 3B1 markedly inhibits the binding of labelled proinsulin to both 1E2 and ANT1 whereas 3B1 did not inhibit binding of this ligand to A6 and 1E2 was ~50-fold less effective than 3B1 at inhibiting binding of proinsulin to ANT1. The other mAb combinations tested (A6-free and ANT1 or 1E2 as the solid phase antibody) displayed no inhibition of proinsulin binding to the solid phase mAb. Figure 5(b) shows that mAb 14B, which is known to be unable to bind human proinsulin on its own (Sobey *et al.*, 1989), was capable of binding the labelled prohormone in the presence of both mAbs A6 and 1E2 and to a much lesser extent with 3B1, but not at all in the presence of ANT1.

Computer modelling of insulin

The surface structures of the mutant insulins were analysed using coordinates provided by X-ray analysis (see Table II). The mutant insulins have not yet been crystallized as monomers but only as dimers or hexamers (depending on conditions). Monomers from these crystal structures and those from native insulin hexamers and dimers were used for studying the structural relationships between the epitopes. Comparisons were made using the program

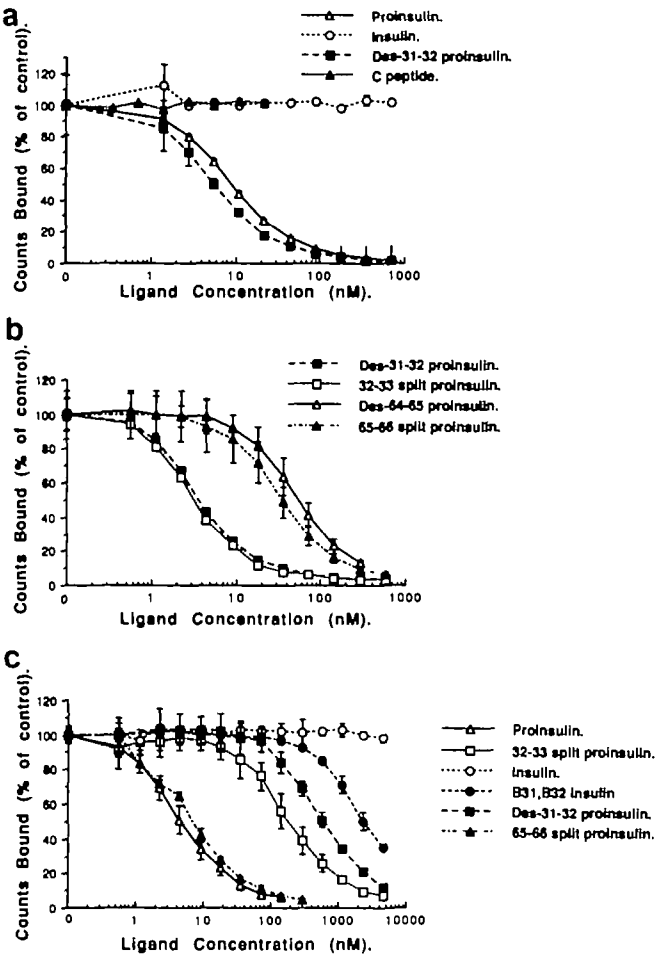


Fig. 4. (a) A des-31–32 proinsulin displacement curve for mAb ANT1. The antibody was incubated with [^{125}I]human des-31–32 proinsulin in the presence of increasing concentrations of unlabelled proinsulin-related species. Proinsulin displacement curves using [^{125}I]human proinsulin for mAb (b) ANT1 and (c) A6. Each point on all the curves represents the mean of four observations ± SEM.

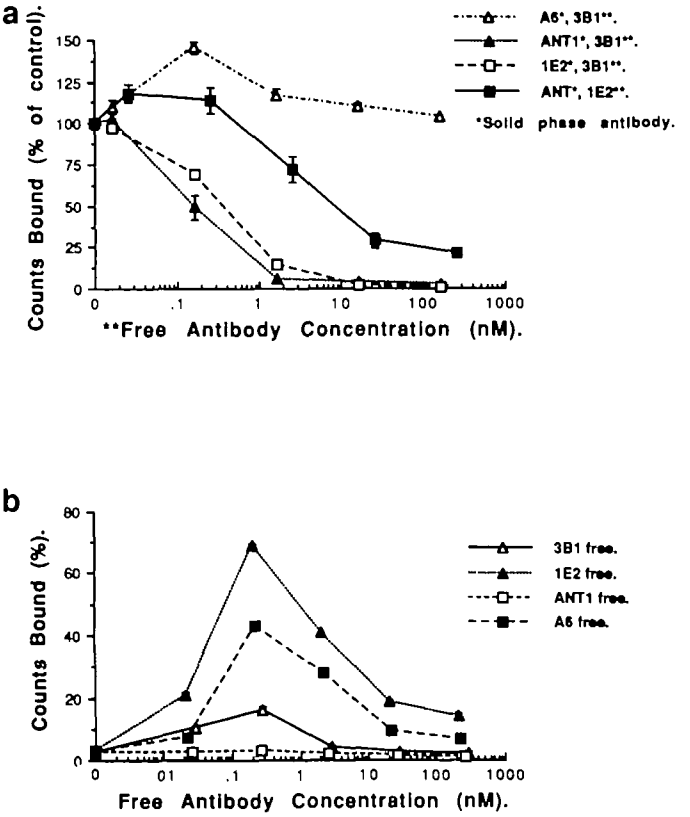


Fig. 5. Antibody competition assays. (a) Microtitre plate wells were coated with mAb (*) and incubated with [^{125}I]human proinsulin in the presence of unbound secondary mAbs (**). (b) Microtitre plate wells were coated with mAb 14B and incubated with [^{125}I]human proinsulin in the presence of unbound secondary mAb. Each point on all the curves represents the mean of four observations ± SEM.

QUANTA on the Unix Silicon Graphics Power Series 4D/380 and the results are shown in Figure 6.

Discussion

Using partially processed proinsulins and insulins both from different animal species and those containing specifically engineered amino acid substitutions we have been able to determine epitopes on human insulin and proinsulin that are recognized by a number of mAbs. Such an analysis does yield useful data although it is necessary to be cautious about its interpretation. The data from the animal insulins are complementary to those obtained from the mutant insulins. The amino acid differences seen in the former are limited to the A-chain loop (A8–A10) and the B-chain C-terminus (B30) and these residues are not important to the hormone's structure, self-assembly or activity (Blundell *et al.*, 1971; Blundell and Wood, 1975). By contrast the changes in the mutant insulins lie in regions where they will modify (generally reduce) self-assembly. Some of the mutations have distinct effects on the hormone's binding affinity to its receptor (Brange *et al.*, 1990). However, analysis of some of the mutant insulins used in these studies by X-ray analysis and other techniques reveals that the chain folding and overall structure are preserved (see Table II). There are however small changes in the contacts between the A- and B-chain C-terminal residues. These changes are sometimes associated with changes in insulin's binding affinity for its receptor (Brange *et al.*, 1988). The structural changes seen in these mutant insulins nonetheless do not appear to affect significantly the organization of the hormones receptor binding surface.

The epitope for 14B is clearly defined by its lowered affinity for bovine, ovine and equine compared to human insulin. Also changes in amino acid residues at positions A4 and A8 and addition of Phe to residue A1 all block binding of 14B to its ligand. These residues also lie very close together on the surface of the insulin molecule (see Figures 6a and 7). Changes at the C-terminal end of the insulin A-chain and the N-terminus of the B-chain have no effect on 14B binding. However, removal of the five C-terminal residues of the B-chain produces an insulin that is bound with greater affinity by 14B than the intact hormone. These residues are known to be very close to the N-terminus of the A-chain (Baker *et al.*, 1988) and, thus, their removal may allow easier access of 14B to its epitope. Furthermore, a mutation at residue B27 converting Thr to Glu produces an insulin that binds to 14B more strongly than normal human insulin. This may be due to the introduction of a charged amino acid causing the flexible C-terminus of the insulin B-chain to move away from the A-chain again allowing 14B greater access to its epitope and this is equivalent to the exposure of the A-chain N-terminus in despentainsulin. The region bound by 14B is known to comprise of a helix extending from residue A2 to A6 and a loop comprising residues A7–A10 (Baker *et al.*, 1988). This latter region has been shown to be highly antigenic according to the criteria of

Table II. Crystallographic parameters of some animal and mutant insulins

Insulin	Space group	Cell parameters (Å)	Resolution (Å)	R_f*	References
Beef	R3	$a = b = 82.5$, $c = 33.9$, $\gamma = 120°$	2.5	–	–
DPI beef	C2	$a = 52.7$, $b = 26.2$, $c = 51.7$, $\beta = 93°$	1.3	0.15	Bi *et al.* (1984)
A8H DPI	C2	$a = 52.6$, $b = 26.0$, $c = 51.6$, $\beta = 93.2°$	1.7	0.15	Brady (1988)
A8H	$P2_12_12_1$	$a = 57.0$, $b = 50.0$, $c = 41.2$	1.8	0.15	Brady (1988)
B9DB27E	C2	$a = 79.4$, $b = 61.1$, $c = 62.0$, $\beta = 95.2°$	1.8	0.18	Turkenburg (1991)
B13Q	R3	$a = b = 80.6$, $c = 37.6$, $\beta = 120°$	2.5	0.17	Bentley *et al.* (1992)

The crystal structures were all solved by molecular replacement using the atomic co-ordinates of the native molecule from the most appropriate crystal form.
*$R_f = \Sigma[|Fo| - |Fc|]/\Sigma|Fo|$ where $|Fo|$ and $|Fc|$ are the measured and calculated structure amplitudes respectively.
The positional accuracy of the protein atoms in these structures ranges from 0.1 Å for well defined atoms to 0.5 Å for poorly defined atoms.

hydrophilicity and atomic temperature factors (Sasaki *et al.*, 1988).

As demonstrated in Figures 6 and 7 the mAbs 3B1 and 1E2 recognize overlapping epitopes on the insulin B-chain. Data from experiments using the mutant and chemically modified insulins show that changes at residues B1, B3, B10 and B13 of the human hormone reduce the ability of 1E2 to bind insulin. Furthermore, computer modelling showed that these residues are clustered together on the surface of the insulin molecule. Mutation at residue B16 of insulin had no effect on 1E2 binding. For 3B1, mutations at residues B10, B13 and B16 of insulin reduce the affinity of the mAb for its ligand and these residues are also grouped together on the insulin surface (see Figures 6b and 7). Changes at B3 had no effect on 3B1 binding. An insulin with mutations at residues B1 and B27, however, was found to bind 3B1 more effectively than the human hormone. Also, insulins with mutations at residues B9 and B21 were very slightly better at displacing the labelled hormone from the mAb compared to human insulin. Thus, residues B1, B9 and B21 must be close to the proposed 3B1 epitope and examination of human insulin structure shows this to be the case. The mAb 1E2 also binds the double-mutated insulin B9D,B27E more strongly than human insulin and again the B9 residue is very close to the proposed 1E2 epitope. The overlapping nature of these epitopes is further demonstrated by the inability of 1E2 to bind insulin (data not shown) or proinsulin in the presence of 3B1. The epitope bound by 3B1 includes the B-chain α-helix that runs from residue B9 to B19 (Adams *et al.*, 1969) whilst the 1E2 epitope spans a region of insulin that can adopt two different conformations. These are the extended or T-conformations, as first described in porcine 2-zinc insulin crystals by Adams *et al.* (1969) and the α-helical or R-conformations as first described in porcine 4-zinc insulin crystals by Bentley *et al.* (1976). From modelling (see Figure 6c and d) it seems likely that either the R- or the T-structures could be bound by the mAb. In both conformations the B1 and B3 residues are relatively mobile and could easily contact the antibody binding surface. Both mAbs 3B1 and 1E2 are able to bind human proinsulin with affinities very similar to those with which they bind human insulin. Thus, the epitopes recognized by these antibodies must be accessible to them on proinsulin and, therefore, are not obscured by the C-peptide moiety. This also demonstrates that the insulin portion of proinsulin is very similar in structure to that of the free insulin molecule as has been demonstrated by other investigators (Frank *et al.*, 1972; Weiss *et al.*, 1990).

The data shown in Figure 4(a and b) suggest that ANT1 binds across the C−A junction and A6 across the B−C junction of intact proinsulin. Thus, neither of the antibodies are able to bind insulin or C-peptide. Also, ANT1 binds des-31−32 and 32−33 split proinsulin to an equal degree but has a lowered affinity for the proinsulin C−A junction cleavage products; the split derivative is slightly better at displacing proinsulin from ANT1 compared to the des product. The converse is true for mAb A6 which binds equally well to intact proinsulin and 65−66 split proinsulin but has a much lower affinity for the proinsulin B−C junction cleavage products. Also addition of two arginine residues onto the C-terminus of the insulin B-chain produces a molecule which, at high concentrations, is capable of displacing proinsulin from A6. It is interesting that mAbs ANT1 and A6 are still able to bind their respective epitopes (although with 10- and 30-fold reductions in affinity respectively) even after the epitopes have undergone cleavage. This suggests that endopeptidase processing of proinsulin results in intermediates in which the newly formed N- and C-termini are able to come close enough together to be recognized by an antibody. This in turn suggests that C-peptide may be interacting with the insulin moiety of the proinsulin molecule in the region of the C−A and B−C junctions and that these interactions may be maintained after cleavage has occurred at these sites on the prohormone. Evidence for such interactions existing at the intact C−A junction of proinsulin comes from the data for 14B. Results of competitive ligand-binding assays showed that insulin and des-64−65 proinsulin at concentrations of 12.5 nM and 24 nM respectively could reduce the level of [^{125}I]insulin bound by 14B to 50% of that of the control binding (control binding is defined as the level of [^{125}I]insulin bound by 14B in the absence of any competing ligand). Both ligands lack the intact C−A junction whilst des-64−65 proinsulin has an intact B−C junction. However, proinsulin and des-31−32 proinsulin both of which contain intact C−A junctions, will only displace 50% of the [^{125}I]insulin from 14B at concentrations of 640 nM and 540 nM respectively (in all experiments 14B was used at a concentration of 1.4 nM and [^{125}I]insulin at a concentration of 9.0 pM). Sobey *et al.* (1989) reported similar ligand binding properties for 14B. This must mean that the intact B−C junction region (see Figure 7) does not interfere with 14B binding but the C−A junction structure does. The fact that des-64−65 proinsulin is half as effective as insulin in displacing [^{125}I]human insulin from 14B may be due to the existence of a weak interaction between the N-terminus of the insulin A-chain and the C-terminus of the C-peptide moiety of des-64−65 proinsulin which interferes with 14Bs ability to bind its epitope. The presence of an interaction between insulin and C-peptide at the C−A region of proinsulin has also been demonstrated by Weiss *et al.* (1990). Their NMR analysis of proinsulin and its cleavage products defined a stable C−A junction structure that was suggested to be a recognition element for the type II endopeptidase. The

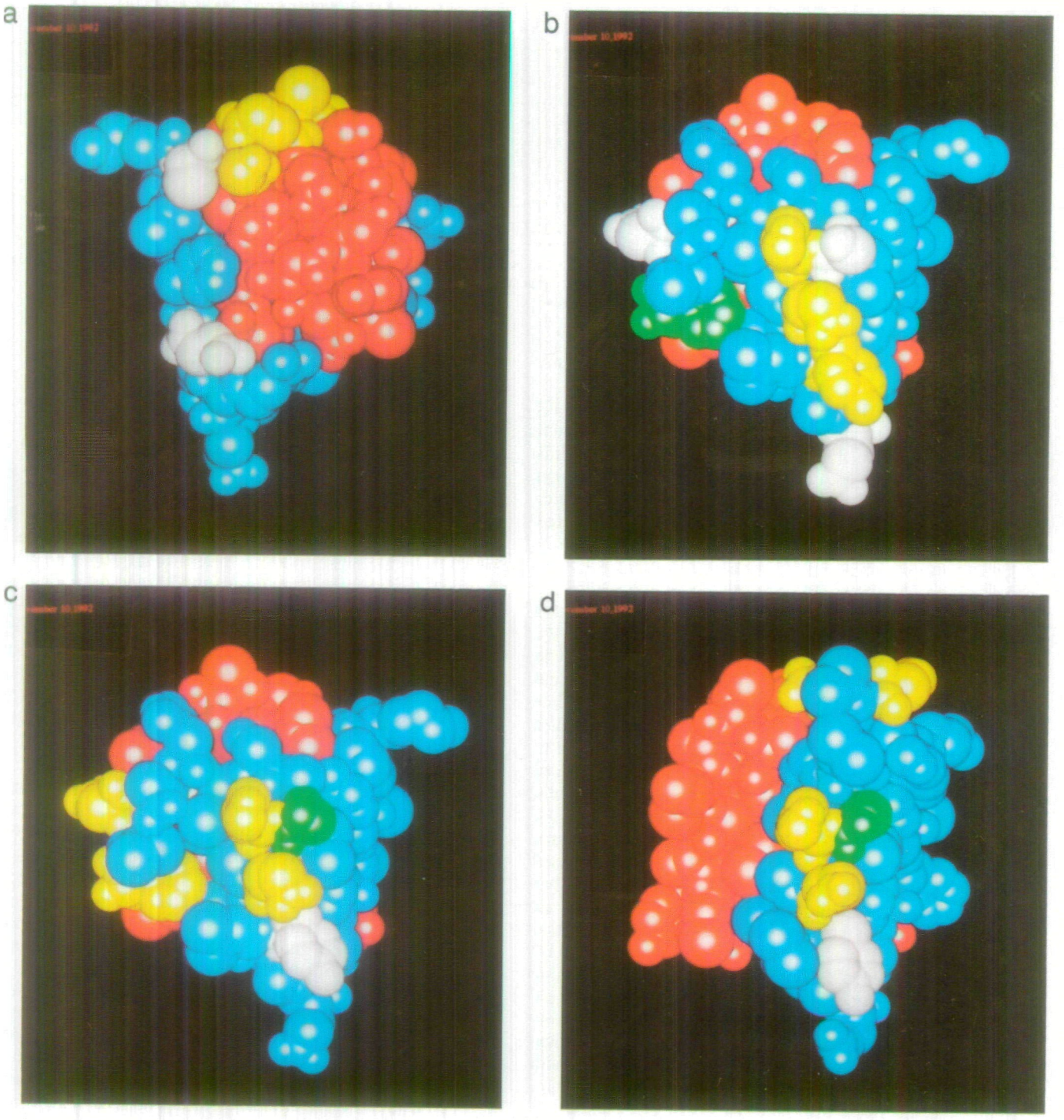


Fig. 6. (a) Insulin in the T-state and viewed down the dimer interface. Amino acids within the 14B epitope, A1, A4 and A8, are shown in yellow. Residues in white, A21 and B30, are not in the epitope. (b) Insulin in the T-state and viewed perpendicular to the dimer interface. Amino acids within the 3B1 epitope, B10, B13 and B16 are in yellow. Residues in white, B3, B9 and B21, are not in the epitope. Residue B1, shown in green, when mutated produces an insulin that binds more tightly to 3B1 than the human insulin. Insulin in the (c) T- and (d) R-states viewed perpendicular to the dimer interface. Amino acids within the 1E2 epitope, B1, B3, B10 and B13, are in yellow. The residue in white, B16 is not in the epitope. Residue B9, shown in green, when mutated produces an insulin that binds more tightly to 1E2 than the human hormone. In (a)–(d) the insulin A-chain is shown in red and the insulin B-chain is shown in blue.

greater loss in affinity of A6 for its epitope after cleavage at the B–C junction suggests that any interaction between insulin and C-peptide in this region is less than that at the C–A junction. This is consistent with the data of Weiss *et al.* (1990) and the known flexibility of the C-terminus of the insulin B-chain (Dodson *et al.*, 1979; Baker *et al.*, 1988). It should be noted that the exact structure of the C-peptide portion of proinsulin is not known and, therefore, the nature of the interactions between insulin and C-peptide are a matter of conjecture. However, models of insulin structure have been produced based on theoretical considerations. Thus, Snell and Smyth (1975) studied the sequence of 10 mammalian C-peptides and used this to predict the 3-D structure of the C-peptide moiety of the prohormone. They identified conserved residues at both the C–A and B–C junctions and

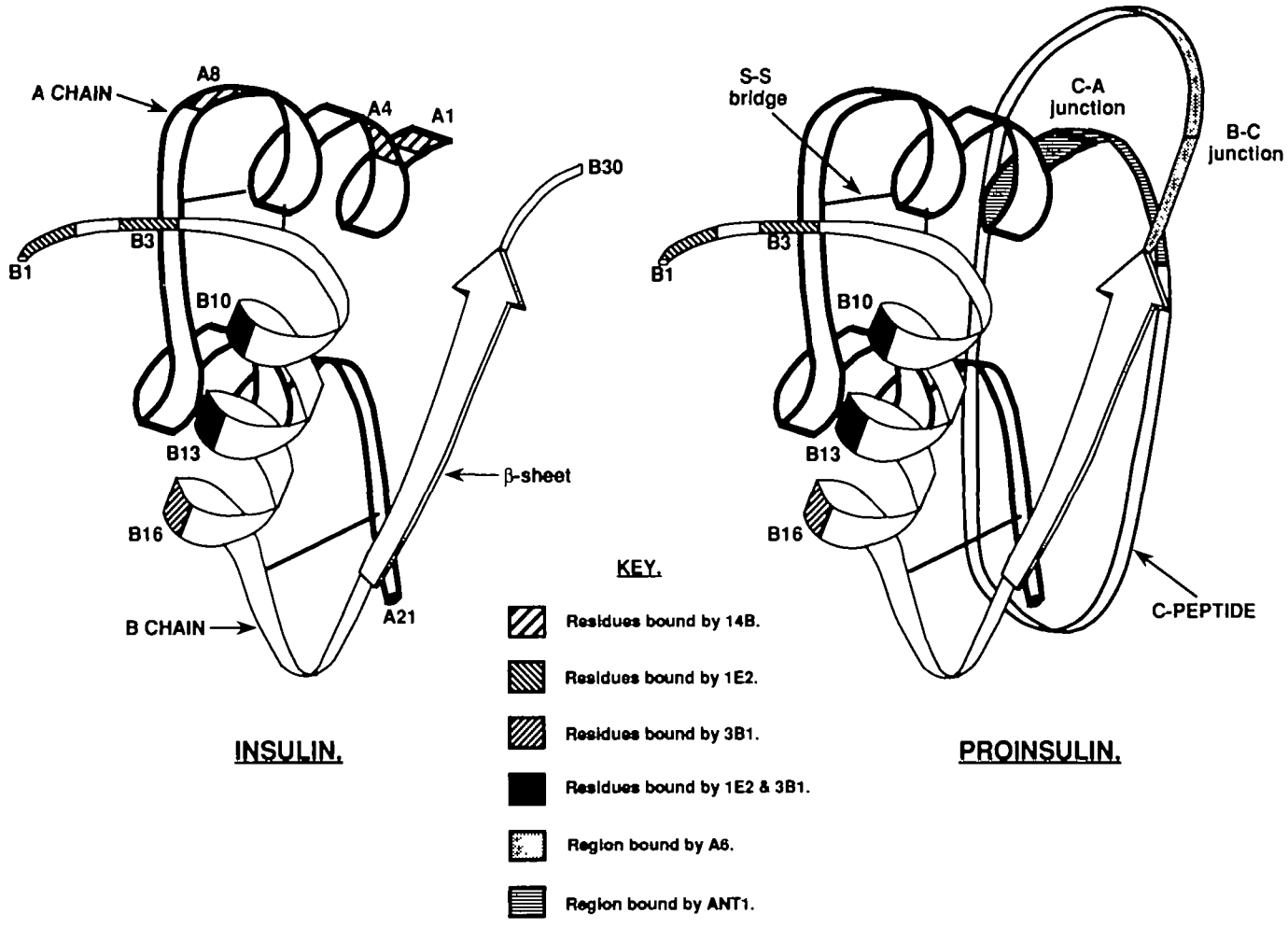


Fig. 7. Ribbon diagram of the T-conformation of insulin and proinsulin showing regions bound by five mAbs. The exact residues bound by mAbs ANT1 and A6 are not known therefore regions around their proposed epitopes, the C−A junction and B−C junction respectively, are shaded. The 3-D structure of the C-peptide moiety of proinsulin is not known and, therefore, the proinsulin model depicted above shows the structure proposed by Blundell *et al.* (1978).

postulated that these may be involved in electrostatic interactions with residues on the insulin moiety. The proinsulin structure depicted in Figure 7 is based on that produced by Blundell *et al.* (1978) which in turn was derived from the work of Snell and Smyth (1975).

Our epitope mapping data were supplemented by competition assays using our mAbs and radiolabelled proinsulin. These studies revealed some unexpected observations. It appears that ANT1 binds proinsulin very poorly in the presence of 3B1 even though their epitopes are separate on the surface of the prohormone. This observation suggests that when 3B1 binds at its proinsulin epitope the mAb disturbs the conformation of the connecting peptide which affects the structure presented at the C−A junction. In contrasting behaviour the mAb 14B, which is known to be unable to bind proinsulin alone, will bind the prohormone in the presence of the mAb 1E2 and in the presence of antibody A6, as also shown by Sobey *et al.* (1989). Thus, binding of 1E2 to proinsulin is associated with a major conformational change in prohormone structure, possibly involving disruption of the C−A junction interactions described above. One possible mechanism by which 1E2 could bring this about would be via stabilization of the R-conformation. In this structure the B1−B8 region is adjacent to the insulin A-chain N-terminus therefore possibly disturbing the interaction between the C-peptide and this region of the insulin moiety of proinsulin. In the case of A6, whose binding to proinsulin allows 14B to bind also, its epitope (the B−C junction) is only 10 Å distant from the C−A junction (Blundell *et al.*, 1972) and, thus, it is feasible that A6 binding could cause a local conformational change within the connecting peptide that would allow 14B access to its epitope.

The data from the epitope mapping studies demonstrated that the mAb ANT1 recognized an epitope that was common to intact, des-31−32- and 32−33 split proinsulin whilst the mAb 1E2 bound an epitope common to insulin, intact proinsulin and all the proinsulin cleavage products. However, in an assay format using ANT1 conjugated to aminocellulose as the primary capture antibody and free radiolabelled 1E2 as the secondary signal antibody, detection of proinsulin compared to 32−33 split proinsulin was poor. Thus, the assay detected 30 pM 32−33 split proinsulin and 135 pM proinsulin (mean + 2 SD of the zero signal). This combination of mAb must therefore be able to discriminate between intact proinsulin and proinsulin that has been cut at the B−C junction. Interestingly, competition assays using radiolabelled proinsulin and microtitre plate-bound ANT1 demonstrated that proinsulin binding to this mAb was partially inhibited by 1E2 when present in nanomolar concentrations (see Figure 5a). The maximum level of 32−33 split proinsulin in human serum following administration of a glucose load is at best two-thirds that of the lowest level of the split proinsulin (30 pM) that our assay can measure (Sobey *et al.*, 1989; Clark

et al., 1992). However, we have found that the assay sensitivity can be improved with the use of fluorescent labels which increase the sp. act. of the signal antibody (1E2) and lower the background reading. Such a 32–33 split proinsulin assay would be of importance since earlier studies, using assays that cross-react with proinsulin, have shown that the level of 32–33 split proinsulin in the sera of type II diabetics and individuals with impaired glucose tolerance is elevated with respect to control subjects (Temple *et al.*, 1989; Williams *et al.*, 1991).

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