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Insulin assembly: its modification by protein engineering and ligand binding

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X-ray analysis of insulin crystals has revealed the nature of the surfaces involved in its assembly to dimers and hexamers. The protein contacts between monomers are well defined but can vary. Contacts between dimers in the hexamer are generally looser and can change remarkably in their structure, particularly by the existence of extended or helical conformations at the N terminus of the B chain.

The assembly of insulin to hexamers is associated with the hormone's slow absorption by tissue; in the diabetic this can lead to inappropriate insulin levels in the blood. Experiments to improve insulin absorption at the injection site have been based on constructing 'monomeric' insulins by protein engineering. These have led to stable monomers with more rapid absorption characteristics. The most effective mechanism to favour the monomeric state was the introduction of carboxylic acids which generated electrostatic repulsion in the dimer and hexamer species. Some of the mutated insulins have been crystallized and their structures determined, revealing the structural basis of their assembly properties.

In the presence of chloride or phenol (and related molecules) the otherwise extended structure of residues B1–B8 forms an alpha helix, packing against the adjacent dimer. This provides additional sites for zinc at the dimer–dimer interfaces, and also can provide a binding site for phenol and related molecules. The surfaces in this cavity provide a template for modelling in other ligands.

1. Introduction

The association of proteins into oligomers and polymers is immensely important in extending their capacities to do chemistry, to adapt to their biochemical environment and to provide structural elements in cells and organs. A great many subunit structures are now determined and some generalizations are emerging about their stabilizing interactions. Analysis of the protein–protein contacts between subunits reveals that their assembly is guided and stabilized by salt bridges, H-bonds, dispersion forces and by the entropic benefit associated with water released by the burial of non-polar surfaces. The specificity of the subunit contacts is essential for effective assembly and the interactions that govern it illustrate splendidly the mechanisms and importance of molecular recognition.

The insulin molecule and its precursor, proinsulin, assembled to dimers and, in the presence of zinc, to hexamers (Grant *et al.* 1972; Blundell *et al.* 1972; Mark & Jeffrey 1990). This behaviour is inherited from the hormone's biosynthesis in the B-cell of

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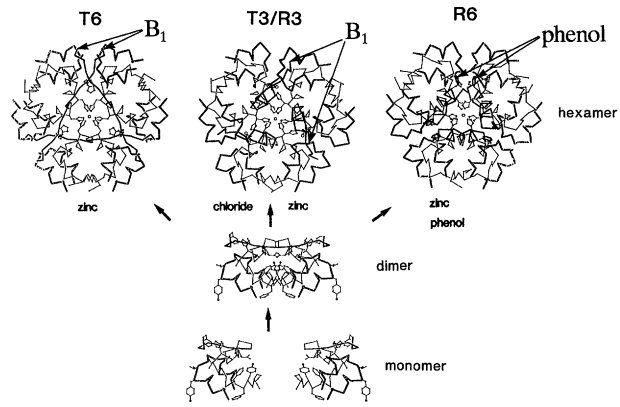

Figure 1. The assembly of the insulin monomers and dimers viewed in the direction of the hexamer three-fold axis. In the dimerization step the buried residues are shown. In hexamer formation only B5 and B10 are shown and at the centre of the hexamers on the three-fold axis the two overlapping zinc sites represented as an open circle. The B1–B8 segments are drawn with thick lines to highlight their structural changes in the different hexamers. The B1 residue is indicated for two molecules adjacent in the dimer, their relative movements can be seen.

the islets of Langerhans (Steiner 1976). In these cells, which are rich in zinc and calcium, the single chain insulin precursor molecule, proinsulin, forms very soluble zinc-containing hexamers (Hermann 1973). This solubility facilitates proinsulin's transport through the Golgi apparatus and its containment in the storage vesicle at high concentrations. As the vesicle buds off from the Golgi the connecting peptide between the insulin A and B chains is removed enzymatically from the soluble proinsulin hexamer to form an insulin hexamer which is insoluble in the presence of zinc. The insulin hexamer typically forms microcrystals in the vesicle which favours efficient conversion (Emdin *et al.* 1980). Thus the hexameric assembly of insulin has enlarged the solution and chemical properties of proinsulin and insulin with great advantages for the biosynthetic processes.

Insulin's aggregation proves to be an important factor in its therapeutic effectiveness since it appears to limit the rate subcutaneously injected insulin is absorbed by the tissues. This effect of sluggish absorption is reduced by insulins that have been mutated to stabilize the monomeric state demonstrating the potential for protein engineering to allow and to improve the pharmacological use of proteins (Brange *et al.* 1988; Kang *et al.* 1991).

2. Insulin structure and assembly

Insulin's three-dimensional structure was determined by X-ray analysis as a 2Zn insulin hexamer (Adams *et al.* 1969). It was seen that the molecule's A and B chains were arranged compactly around a non-polar core made up of the central B-chain helix B9–B20 and the inner directed residues from the two short, adjacent and anti-parallel A-chain helices A1–A8 and A13–A20. There prove to be two non-polar surfaces on the molecule, one involved in dimer assembly, the other in hexamer assembly. The knowledge of the crystal structure and the large body of solution and biochemical observations means that insulin's pattern of assembly is well understood in structural and chemical terms (Blundell *et al.* 1972). It is illustrated in figure 1. The dimer is constructed from two two-fold related molecules whose association is

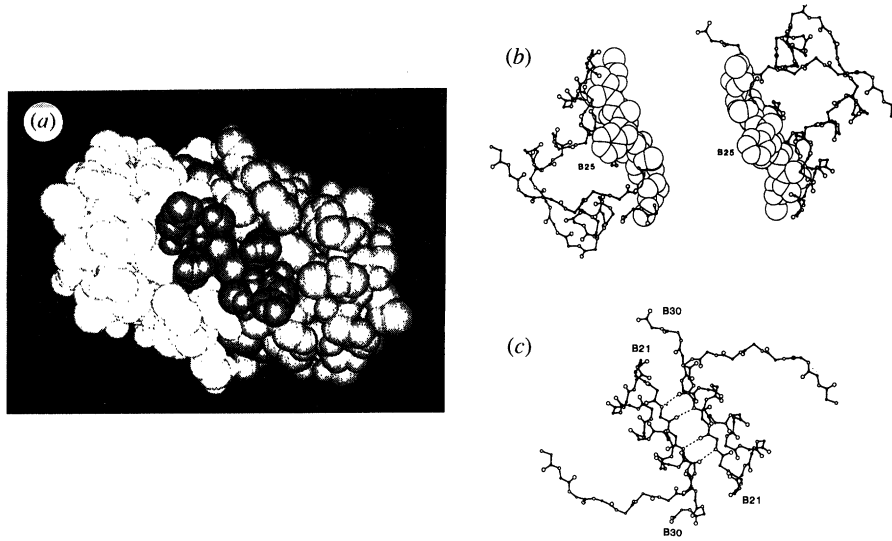

Figure 2. The insulin dimer viewed in the direction of its two-fold axis. (*a*) Atoms are given their van der Waals radius and one monomer is shaded more lightly than the other. In this view the mutated residues B9 serine, B10 histidine, B13 glutamic acid and B28 proline are in front, they are shaded dark. (*b*) The dimer-forming surfaces seen from the opposite side of the dimer; the dimer-forming residues are shown with van der Waals radii. The dimer shown here is from the T6, 2Zn insulin hexamer in which the two equivalent B25 phenylalanine rings take different conformations (Baker *et al.* 1988). (*c*) shows the anti-parallel H-bonded B-sheet which is formed by the B-chain C-terminal mainchain between the monomers (Baker *et al.* 1988).

stabilized by the burial of two equivalent non-polar and mostly aromatic surfaces whose packing together is seen in figure 2*a*. At one edge of these surfaces is the B-chain C-terminal segment of some 10 residues. These run antiparallel and come together between B24 and B26 to form a B-sheet H-bonded structure which adds further compactness and specificity to the dimer forming interactions (figure 2*b*, *c*). Examination of these dimerising surfaces from different insulin dimers and hexamers (which are constructed from dimers) shows that although they make close and complementary non-polar contacts they are relatively flexible and can accommodate some conformational changes in the sidechains. For example insulin dimers crystallized at low and neutral pH show distinct variations in some of the non-polar contacts at the interface. A comparison of these contacts helps explain the surprisingly limited effects that the introduction of extra non-polar groups at the monomer–monomer interface has on dimer and hexamer formation (see below).

3. Insulin hexamer formation

The assembly of insulin dimers to hexamers seen in figure 1 requires the coordination of zinc ions (or other divalent metal ions) to the B10 histidine imidazole rings. Each of the three equivalent dimers presents two B10 histidine sidechains to the zinc ions which lie on the central three-fold axis. The dimers make looser contacts in the hexamer than the monomers in the dimer though like the dimer they pack together around a central non-polar region, see figure 3. There are polar and charged residues clustered together in the central channel of the hexamer all associated with extensive solvent structure. At the very centre lie six glutamic acid sidechains.

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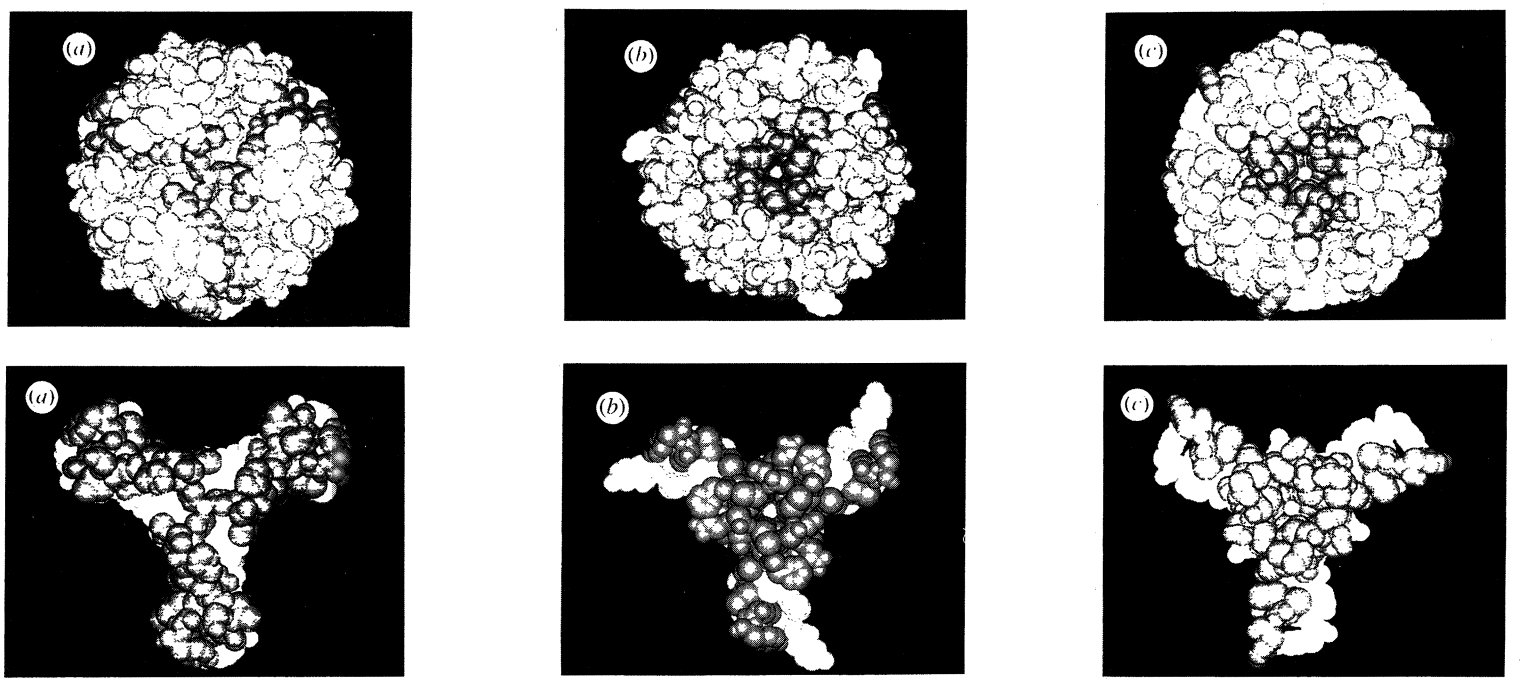

Figure 3. The zinc containing hexamers shown in the upper panel and the envelopes of contacts between the dimers shown in the lower panel viewed in the direction of their three-fold axis. All atoms are drawn as van der Waals spheres. The zinc ions lie overlapped on the central three-fold axis. Contacts between dimers arising from the top trimer are differentiated from those from the lower trimer by darker shading. Other atoms are given intermediate shading. (*a*) is the T6 hexamer; note the extensive contacts and the obvious presence of the local two-fold axis in the envelope of contact. (*b*) is the T3R3 hexamer; there are fewer contacts and an absence of symmetry in the envelope. The new contacts between the helical residues are developed about the three-fold axis partly burying the zinc. (*c*) is the R6 hexamer. Note the further reduction in the envelope of contacts between the dimers. This figure was produced using QUANTA (MSI).

Normally these residues would be charged and their proximity is unfavourable for the hexamer's stability (Baker *et al.* 1988; Bentley *et al.* 1992).

Crystallographic studies have now identified three distinct hexamer structures which differ principally in the conformation at the B1–B8 residues. These are illustrated in figure 1 and in figure 3. When the B1–B8 segment (indicated in the figure 1) is extended, the molecule is described as T and the hexamer as T6 (Kaarsholm *et al.* 1989; Derewenda *et al.* 1989). In this structure the B1–B8 residues cross over between dimers and bury the non-polar B1 phe and B2 val in a non-polar pocket in the adjacent dimer (Baker *et al.* 1988). The T6 hexamer has approximate 32 point symmetry and the dimer–dimer contacts obey twofold symmetry well, as can be seen in figure 3*a*.

The B1–B8 segment forms helix in the second kind of hexamer and becomes a continuation of the B9 to B19 helix which is a central feature in all insulin molecules. This conformation, referred to as the R structure, moves the N-terminal residues by 20–30 Å. When insulin is grown from high chloride ion concentrations the hexamer contains three molecules in the T state and three in the R state; this is described as T3R3 (again see figure 1). The local symmetry between the dimers is completely abolished in this hexamer, as can be observed in figure 1 and figure 3*b*. There are still many contacts between dimers in the hexamer but only one B1–B8 segment is involved. The helical B1–B8 residues form new interactions between themselves; B6 leu in particular forms a non-polar cluster with its two equivalents around the three-fold axis. This interaction closes off the zinc from solution and reduces its capacity to exchange significantly, thus increasing hexamer stability (Kaarsholm *et al.* 1989). In addition the B5 his is now available to form with a re-oriented B10 his a second zinc coordination site, off the three-fold axis. When occupied this site provides an alternative mechanism for stabilizing the hexamer (Bentley *et al.* 1976; Smith *et al.* 1984).

More recently the structure of a zinc insulin hexamer has been determined in crystals grown in the presence of phenol (Derewenda *et al.* 1989; Smith *et al.* 1992). In this hexamer all six molecules are helical at B1–B8, it is designated as R6 and has 32 symmetry. The contacts between two-fold related dimers are reduced further and are limited to the non-polar residues B14, B17, B18 and A13, as is illustrated in figure 3*c*. There are now non-polar interactions between the helical B6 leucines outside both axial zinc sites further increasing the hexamer's stability. The phenol molecules bind specifically between dimers and sit on the potential off-axial zinc site. They form favourable H-bonds through the OH function with the mainchain A6 carbonyl oxygen and the A11 peptide nitrogen and they also make van der Waals interactions with each dimer.

4. Effects of mutations at the associating surfaces on insulin's assembly

A great many mutations have now been carried out on the insulin molecule to investigate the assembly process and to try to design stable monomers (Brange *et al.* 1990; Brems *et al.* 1992). Some of these mutant insulins have been crystallized and their structures determined (Brange *et al.* 1991). A selection of mutational changes which alter the molecule's aggregation properties are shown in figure 2. In this paper the mutations made at B9, B10, B12, B13, B27 and B28 will be discussed in some detail. Some of the mutated insulins are changed at more than one residue (e.g. B9 ser → asp, B27 thr → glu and A8 thr → his; B10 his → asp and B28 pro → asp). The

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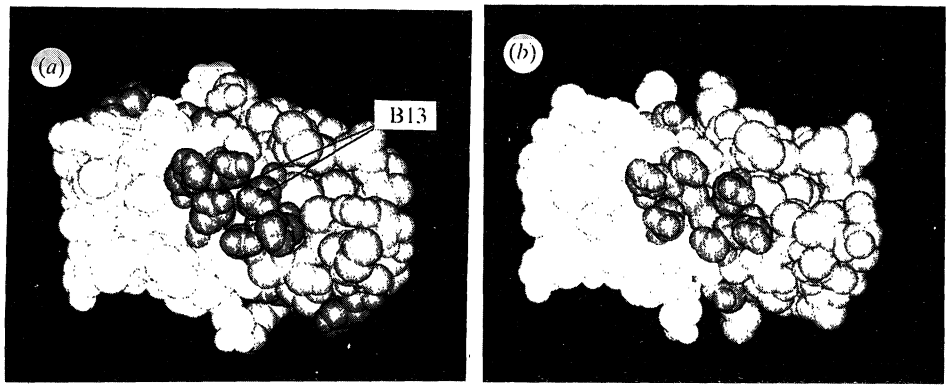

Figure 4. Dimers of (*a*) B9 asp, B27 glu, A8 his insulin and (*b*) B10 asp, B28 asp insulin, viewed down the local two-fold axis. The residues B9, B10, B12, B13, B27 and B28 are shaded in dark grey in both panels, A8 his is also shaded in (*a*) The pair of B13 glu immediately about the local two-fold axis seen in (*a*) are at H-bonding distance in the presence of the B9 asp mutation. In (*b*) the four carboxylic acids are separated and the closest interaction is within the monomer.

Table 1. *Crystallographic and biological information on insulin mutants and liganded insulin hexamers*

insulin	space group	MAU[a]	assembly	Zn	pH	organization	resolution/Å	R_c[b]	potency (%)[c]
pig	R3	2	hexamer	2	6.2	T6	1.5	0.154	100
pig	R3	2	hexamer	4	6.5	T3R3	1.5	0.19	100
B9 asp[d]	C2	6	hexamer	2	6.5	R6	1.8	0.18	31
B9 asp[d]	$P2_12_12_1$	4	dimer	0	6.0	T2	2.0	0.20	116
B10 asp	$P2_13$	4	dodecamer	0	7.3	T12	3.0	0.21	207
B10 asp[d]	C2	2	dimer	0	6.5	T2	2.1	0.19	201
B12 ilu	R3	2	hexamer	2	6.5	T6	2.0	0.16	29
B13 gln	R3	2	hexamer	0	6.4	T3R3	2.5	0.17	33
B13 gln	R3	2	hexamer	2	6.5	T6	2.1	0.19	—
ligand in hexamer									
phenol	$P2_1$	6	hexamer	2	6.5	R6	1.8	0.19	100
phenol	R32	6	hexamer	2	7.8	R6	2.5	0.16	100
paraben	R3	2	hexamer	2	5.4	T3R3	2.0	0.18	100
7-OH-indole	R3	2	hexamer	2	7.0	T3R3	2.7	0.17	100
7-OH-indole	$P2_1$	6	hexamer	2	7.0	R6	3.0	0.20	100

[a] Molecules per asymmetric unit.
[b] $R_c = \Sigma\|\|F_0|-|F_c\|/\Sigma|F_0|$, where $|F_0|$ and $|F_c|$ are the observed and calculated structure amplitudes respectively.
[c] Brange *et al.* (1990).
[d] Principal mutation only (see text).

mutations at B27 and B28 were designed to increase dimer formation; that at A8 was introduced to elevate the molecule's receptor binding affinity. These residues are, except for A8 in, or close to, the interface between the monomers in the dimer. Those at B9, B10 and B13 are also involved in important hexamer forming interactions. Thus these mutations illustrate the different approaches to probing the factors that can govern protein interactions and molecular recognition. Table 1 lists the crystallographic and some biological information on these insulins.

5. Dimer formation

(*a*) *B9 ser → asp*

The B9 asp mutation is one of the most effective in reducing dimer formation at neutral pH (Brange *et al.* 1988). Examination of the dimer structure in figure 2 shows why this is. The B9 ser and B13 glu are spatially adjacent in α-helix; in the dimer they form a closely organized quartet. Thus mutation of the B9 to aspartic acid (which is less flexible than glutamic acid) will introduce strong electrostatic repulsion. The structure of insulin mutated at B9 to asp, B27 to glu and A8 to his has been very recently determined and refined from crystals grown from highly concentrated solutions at pH 6.0 (see table 1). The crystal structure is illustrated in figure 4*a*. It can be seen that there is a close approach of the B13 sidechains. This suggests the carboxylated groups are protonated and H-bonded. This possibility is supported by NMR studies which suggest that B13 glu has an elevated pK in the B9 asp mutant insulin (Kaarsholm *et al.* 1990). At more neutral pH the B13 glu would be ionized and drive the monomers apart.

(*b*) *B10 his → asp*

The B10 and B13 sidechains are quite separated in the native dimer, as can be seen in figure 2*a* (Badger *et al.* 1991). It is not surprising therefore that the B10 his → asp mutation reduces dimer formation less than the B9 ser → asp mutation (Brange *et al.* 1991). The three-dimensional structure of the double mutant insulin B10 asp, B28 asp, which forms a dimer in the crystal, shows that the B13 and B10 carboxylate side groups are reasonably separated and can remain ionized (see figure 4*b*). There will obviously be electrostatic repulsion in this dimer but it is clearly less than in the B9 asp mutant insulin.

The effects of the B28 asp mutation which are significant, occur within the monomers of the dimer. Residues near the asp at B28 have moved away up to an Angstrom and separated the N and C-terminal residues. These movements have not affected the structure of the dimer forming contacts, but will present a structural barrier to their being established.

(*c*) *B12 val → ilu*

The close and specific non-polar interactions made between the monomers described above and shown in figure 2, suggests that dimerization might be prevented by replacing valyl with isoleucyl sidechains. The extra bulk of the two methyl groups however does not greatly reduce the dimer (or hexamer) forming capacity of this mutant (Brange *et al.* 1988). Comparison of the native and B12 val → ile insulin T6 hexamer shows that the interface has adapted to the new methyl groups through a complex pattern of movements. A similar plasticity was also observed in the natural insulin dimer crystallized at low pH.

6. Hexamer formation

(*a*) *B9 ser → asp*

The six central B13 glu sidechains are arranged in the centre of the hexamer where their electrostatic repulsion is a destabilizing feature (Bentley *et al.* 1992). The mutation of B9 to asp increases the cluster of negative charges to 12 and effectively prevents its formation (Brange *et al.* 1988). None the less in the presence of zinc and

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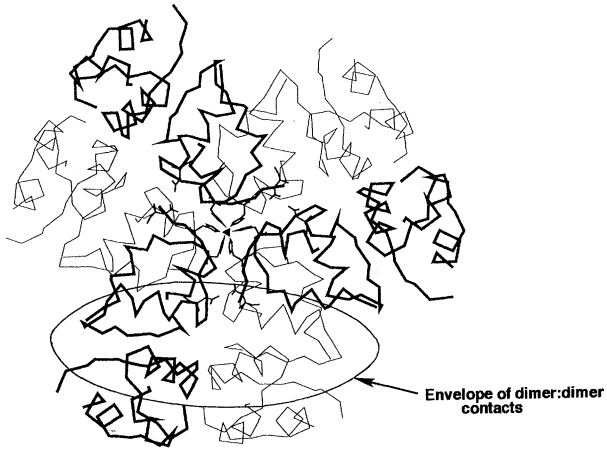

Figure 5. A schematic drawing of the B10 asp insulin dodecamer that occurs in crystals grown in the presence of zinc. C_α positions are shown with peptides represented as lines. The B5 histidines involved in zinc coordination and the mutated B10 aspartic acids from the same monomers are also shown. The two zinc ions on the central three-fold axis are seen as a black triangle. Three dimers are drawn in thick lines and three in thin lines, the region of contact between dimers is indicated.

calcium ions and phenol (originally introduced into the crystallizing medium as an anti-bacterial agent) hexamers of this mutant can be crystallized (Brange *et al.* 1991). The explanation for this entirely unexpected behaviour by the B9 asp mutation was the result first of the fortuitous formation of a calcium binding site by the mutant B9 asp and the natural B13 glu. This interaction cancelled the electrostatic repulsion between these 12 central carboxylate groups. Secondly, the combination of the zinc coordination and the favourable phenol interactions serve to complete the stabilization of an R6 hexamer.

(*b*) *B13 glu → gln*

Hexamer formation can equally be enhanced by removal by the electrostatic repulsion effects generated by B13 glu. This was achieved by mutation of B13 glutamic acid to glutamine which is neutral and which have the capacity to form a ring of linked H-bonds at the hexamer centre. It was found that this mutant insulin formed hexamers in solution in the absence of zinc (Roy *et al.* 1989; Wollmer *et al.* 1989). Crystals of this hexamer have been grown in the absence of zinc from ammonium sulphate. Their X-ray analysis shows them to have the T3R3 organization, demonstrating that the dimer–dimer interactions actually specifically favour the hexamer structure (Bentley *et al.* 1992). It is interesting that the B13 gln sidechains do not interact directly although they can be modelled to do so (Markussen *et al.* 1987).

7. The mutant B10 asp insulin dodecamer

The mutation of B10 his to asp abolishes the hormone's capacity to form a zinc containing hexamer. The molecule, however, still contains a histidine residue at B5. Crystals of the B10 asp mutant insulin can be obtained in the presence of zinc at

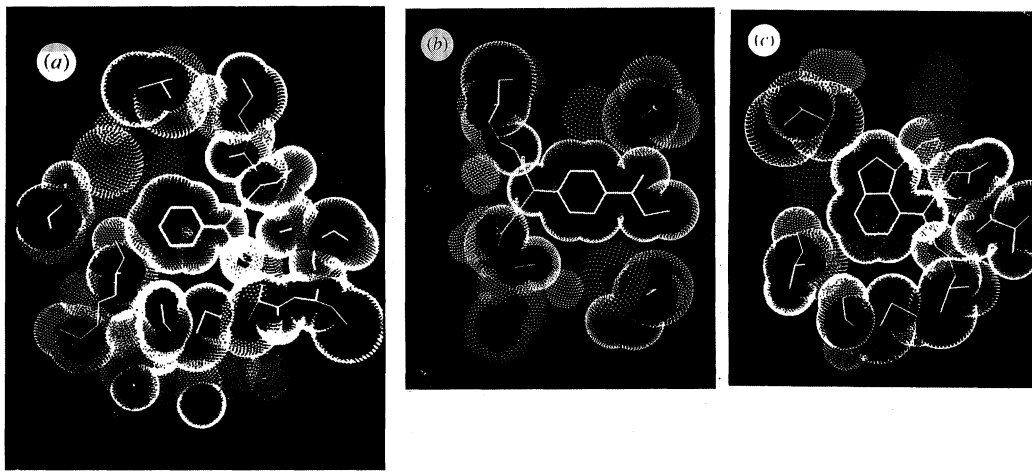

Figure 6. The environment at the phenol binding site in the R state insulin molecule; van der Waal radii are drawn as a dotted surface. The views differ slightly in order to reduce overlap of important structural features. (*a*) Phenol: its H-bonds to the peptide A6 CO and the A11 NH and the close approach of the B5 imidazole ring can be seen. (*b*) Methylparaben: there is an extra H-bond made through the carbonyl oxygen to a water molecule not shown. The methyl ester fills in extra space. (*c*) 7-OH-indole: the additional H-bond made by the indole-N to the A11 CO is made.

neutral pH. Their analysis shows that the insulin molecule is organized as a dodecamer; its structure is seen in figure 5. At its centre on the three-fold axis lie the two zinc ions. These are coordinated to the B5 his imidazole rings, one presented from each dimer. In appearance this coordination is reminiscent of that in the hexamer (Turkenburg 1992).

The dimers in the dodecameric structure are in the T state with the B1–B8 residues in extended conformation and consequently their contacts within this assembly involve the same residues that form the hexamer. The residues on these surfaces however make very different interactions. The interactions that in addition to the B5 his coordination appear critical are three symmetry related complementary H-bond contacts between the B4 glutamine sidechains. Two important residues in hexamer formation are B10 and B13. In the dodecamer within each dimer one B10, mutated to aspartic acid, is salt bridged to the B1-alpha amino group while one B13 glu makes H-bonds to the B9 ser OG and to the B16 tyr OH.

The non-polar surfaces created by B14, B17, B18, A13 and A14 are still loosely congregated together and form a large hydrophobic component in the envelope of contact between the dimers. There is a somewhat similar organization of the B1 phe; one is still internal to A14 tyr but its alpha-amino group is involved in a H-bond to the second B4 gln sidechain oxygen. The other is nearer to the three-fold axis, again its alpha-amino group is internal and as noted above is salt-bridged to the B10 asp. In their general character the dimer–dimer interactions are less sequestered than in the hexamer with polar and non-polar residues occurring along the envelope of contact.

8. The role of phenol and related molecules in the hexamer

It appears that chloride and other Hofmeister species can stabilize only a T3R3

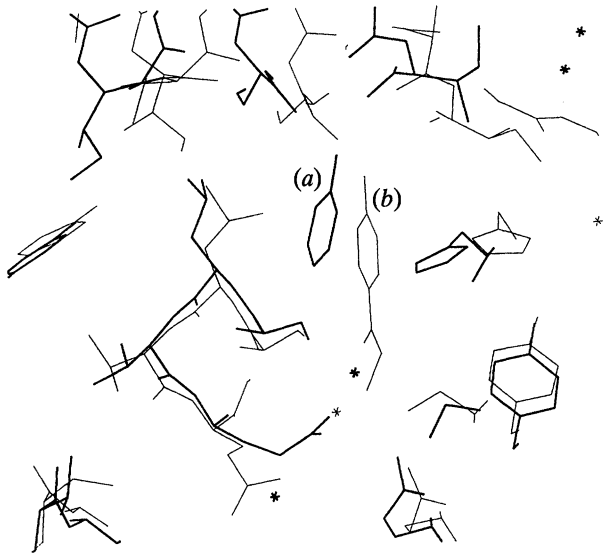

Figure 7. The structural differences between the R6-phenol insulin complex (thick lines) and the R6-methyl-paraben complex (thin lines). The two environments have been overlapped on their B9–B19 helix which have retained their structures. Most of the changes are in the A chain where there are substantial relative movements. (*a*) Phenol, (*b*) *m*-paraben.

hexamer while the presence of phenol is apparently essential for the formation and crystallization of the R6 hexamer (de Graaf *et al.* 1981; Wollmer *et al.* 1987; Kaarsholm *et al.* 1989). Several crystals of T3R3 hexamers have also been grown with methyl-paraben and 7-OH-indole. An overview of the phenol position on the hexamer is shown in figure 1. Inspection of the R6 hexamer crystal structure shows that the phenol OH group is H-bonded to the A6 carbonyl oxygen and to the A11 peptide nitrogen (see figure 6*a*). There are van der Waals contacts between the B5 imidazole and the phenol ring; this contact is with the dimer adjacent to that to which the A-chain contacts are made. Analysis of the phenol contacts showed that there were unused potential H-bonding sites and that there were cavities around the molecule suggesting that substituted phenols might bind even better. This possibility was important since it was a new means of modifying hexamer formation which could lead to improved therapeutic formulations. Modelling experiments and surface calculations identified the indole skeleton as one that could satisfy a further H-bond as well as making more favourable van der Waals contacts than phenol.

A series of binding studies and crystallographic analyses were carried out on phenol, the 7-OH-indole and some phenol related molecules. Their affinities were estimated by spectrophotometric titration using the Co^{II} insulin system which are isomorphous to the Zn^{II}–insulin hexamers (Roy *et al.* 1989). Phenol has low or no affinity to the T6 insulin hexamer but binds readily to the R6 hexamer. The binding of the phenol or ligand to the Co–insulin hexamer in solution therefore switches it from the T6 to the R6 state. This change in hexamer structure is accompanied by a change in the metal coordination from octahedral to tetrahedral which can be followed spectroscopically and can be used to determine the binding characteristics of the phenol and related molecules. The order of affinity in this series was 7-OH-indole → resorcinol → m-cresol → phenol (methyl-paraben discussed below was not determined).

The environments of the phenol, methyl-paraben and 7-OH-indole in the insulin hexamer are shown in figure 6. There are H-bonds to the mainchain A6 carbonyl oxygen and A11 peptide nitrogen. These links are further strengthened in the 7-OH-indole complex by the H-bond to the A11 carbonyl oxygen. The close approaches to the surrounding groups can be seen; there are van der Waals contacts to the B5 imidazole ring which is presented in this way by the helix conformation at B1–B8 and to the B11 leucine. The extra space available about the phenol ring is partly filled by the substituents on the methyl-paraben and 7-OH-indole rings.

There are considerable structural adjustments made by the surrounding insulin sidechains when extra bulk is introduced on the phenol ring; those associated with the methyl-paraben can be seen in figure 7. These changes are much larger than those observed at the monomer–monomer interface and indicate again the more extensive flexibility exhibited by the dimer–dimer contacts. In addition this region is constructed around the ligand as the hexamer switches from the T to R states, which perhaps introduces more apparent flexibility.

9. Conclusions

The interactions that stabilize insulin's assembly prove to be adaptable even when they make close and apparently complementary contacts. This evidently limits the effectiveness of non-polar mutations to perturb self-association. By contrast the electrostatic repulsion generated between carboxylates brought by dimer or hexamer formation proved a successful mechanism which could be readily exploited by protein engineering methods.

It is interesting that the dimer–dimer contacts that are even less compact than those between monomers are sufficiently coherent to form a hexamer without the zinc coordination at B10 his, providing the electrostatic repulsion between the B13 glu residues is removed. The invariability observed in the hexamer contacts is seen in the zinc-free B13 gln hexamer which takes up the T3R3 organization. It follows from these experiments that the B13 glu, invariant as a carboxylic acid in zinc binding insulins, has a functional role: possibly in the rapid dissociation of the hexamer (Bentley *et al.* 1992).

The dodecameric organization of the B10 asp insulin illustrates once again the chemical and structural complexity of the protein surface even in a relatively simple molecule like insulin. These properties give it the capacity to find alternative sets of interactions which include incorporating the mutated sidechain into a salt-bridge contact.

Finally the binding of a series of phenol-related ligands is seen to add extra stability to the hexamer. The binding is associated with substantial movements of sidechains and peptide segments in the near environment but with relatively little movement in the hexamer centre. Here again an intrinsic flexibility is seen in the hormone which parallels the behaviour of the non-polar interfaces. It seems likely that this adaptability reflects a fundamental property of proteins which can be exploited particularly in their recognition of other surfaces.

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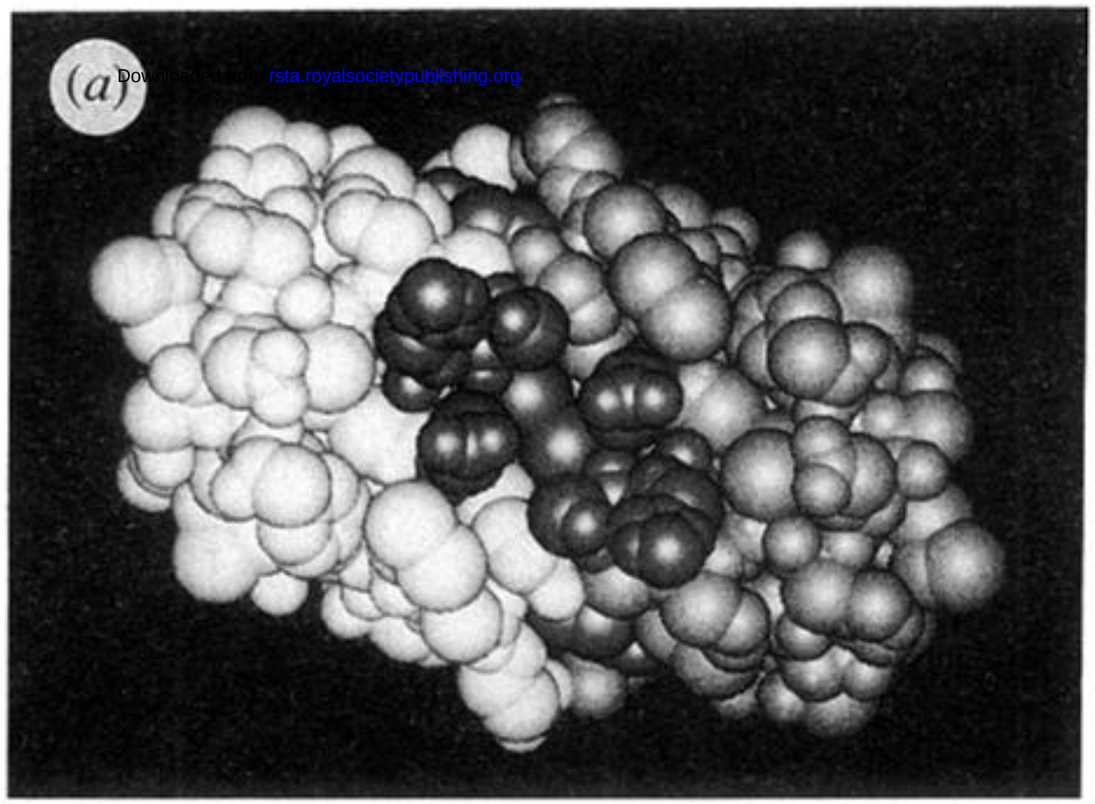


Figure 2. The insulin dimer viewed in the direction of its two-fold axis. (*a*) Atoms are given their van der Waals radius and one monomer is shaded more lightly than the other. In this view the mutated residues B9 serine, B10 histidine, B13 glutamic acid and B28 proline are in front, they are shaded dark. (*b*) The dimer-forming surfaces seen from the opposite side of the dimer; the dimer-forming residues are shown with van der Waals radii. The dimer shown here is from the T6, 2Zn insulin hexamer in which the two equivalent B25 phenylalanine rings take different conformations (Baker *et al.* 1988). (*c*) shows the anti-parallel H-bonded B-sheet which is formed by the B-chain C-terminal mainchain between the monomers (Baker *et al.* 1988).

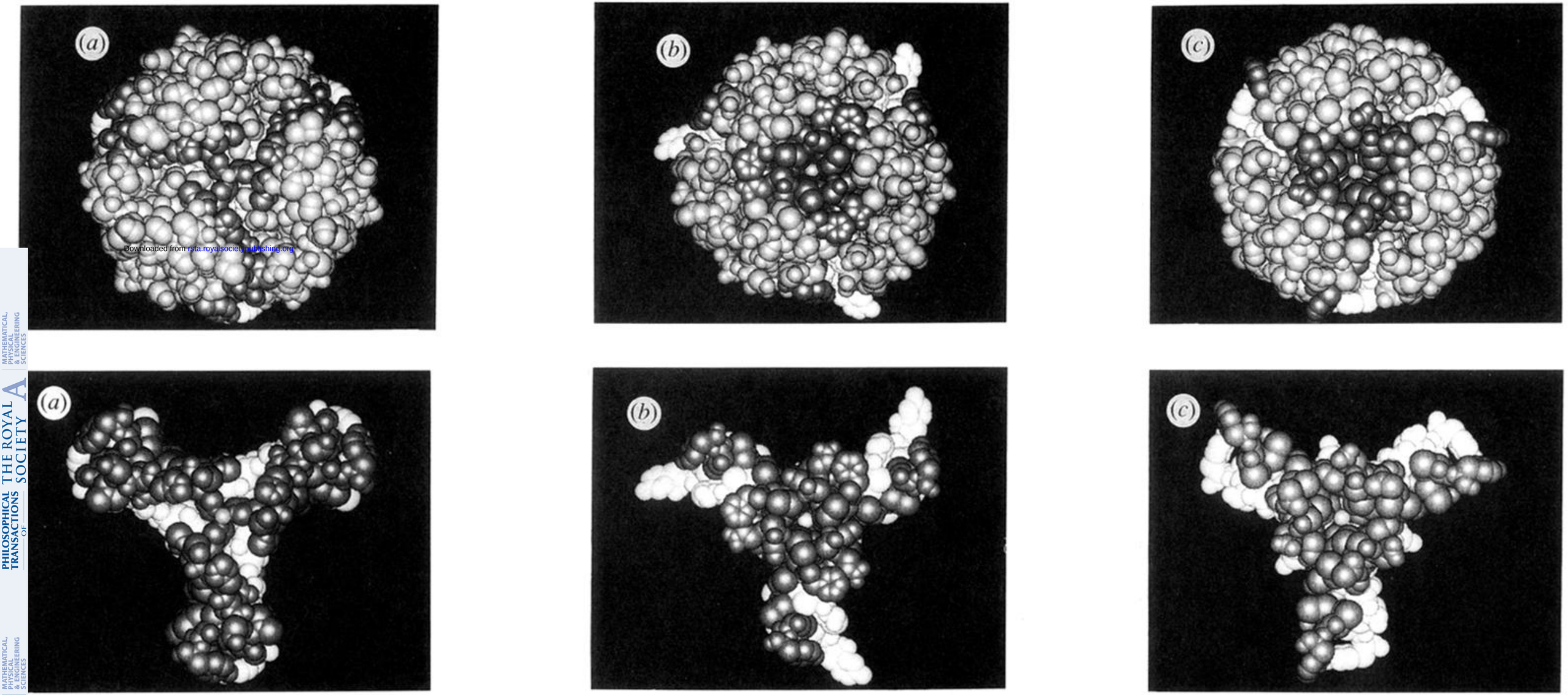


Figure 3. The zinc containing hexamers shown in the upper panel and the envelopes of contacts between the dimers shown in the lower panel viewed in the direction of their three-fold axis. All atoms are drawn as van der Waals spheres. The zinc ions lie overlapped on the central three-fold axis. Contacts between dimers arising from the top trimer are differentiated from those from the lower trimer by darker shading. Other atoms are given intermediate shading. (*a*) is the T6 hexamer; note the extensive contacts and the obvious presence of the local two-fold axis in the envelope of contact. (*b*) is the T3R3 hexamer; there are fewer contacts and an absence of symmetry in the envelope. The new contacts between the helical residues are developed about the three-fold axis partly burying the zinc. (*c*) is the R6 hexamer. Note the further reduction in the envelope of contacts between the dimers. This figure was produced using QUANTA (MSI).

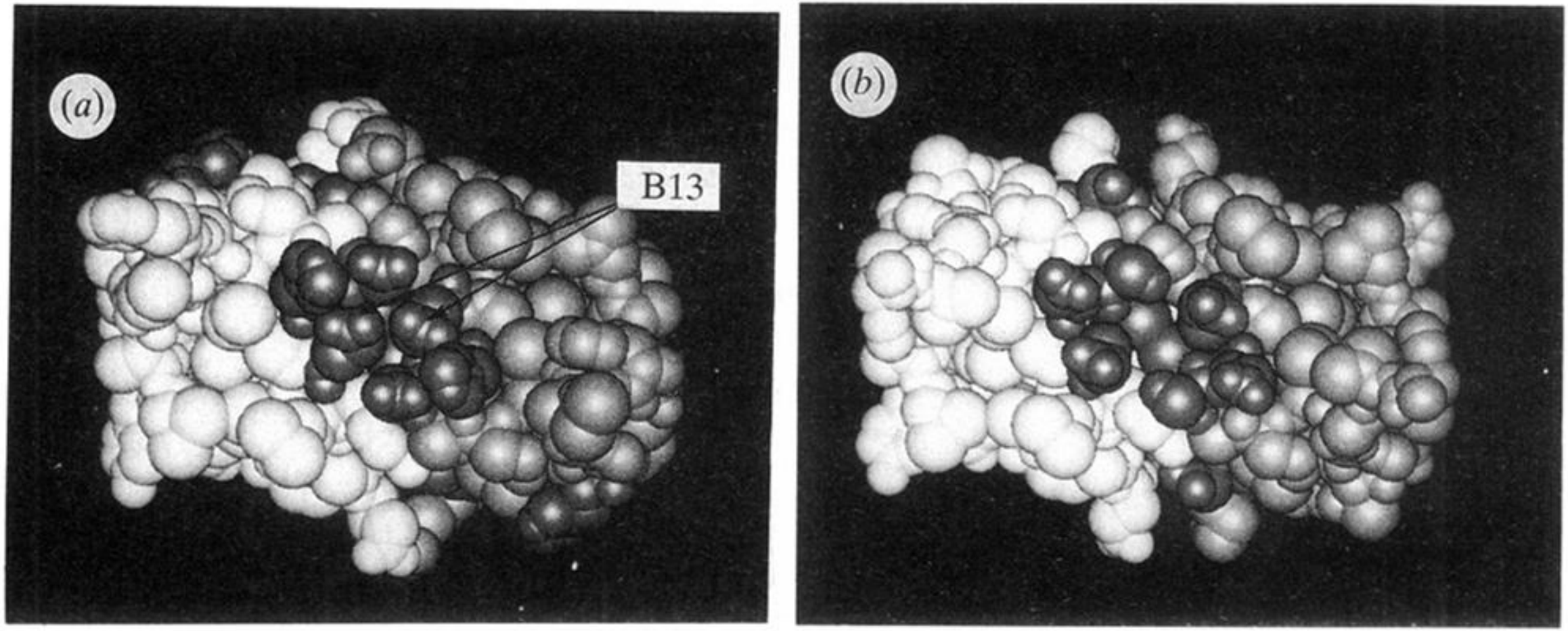
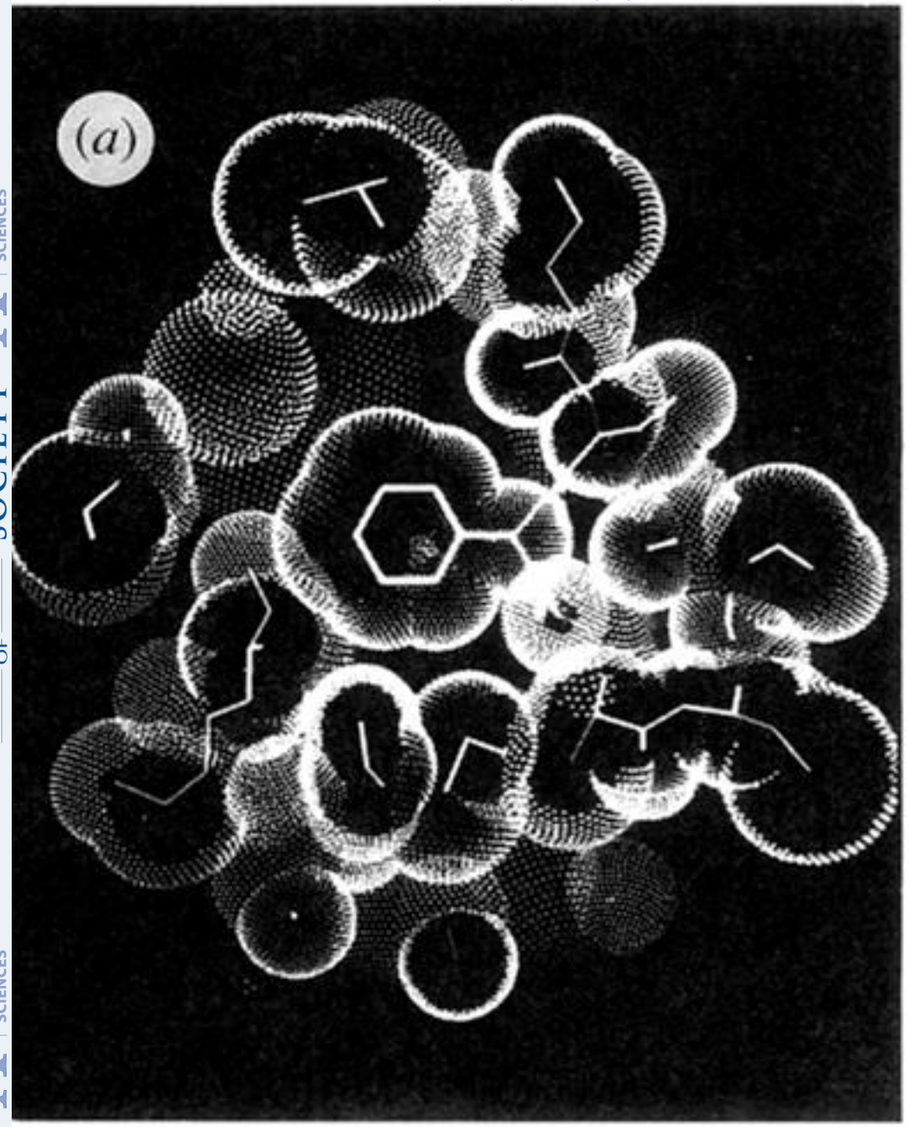
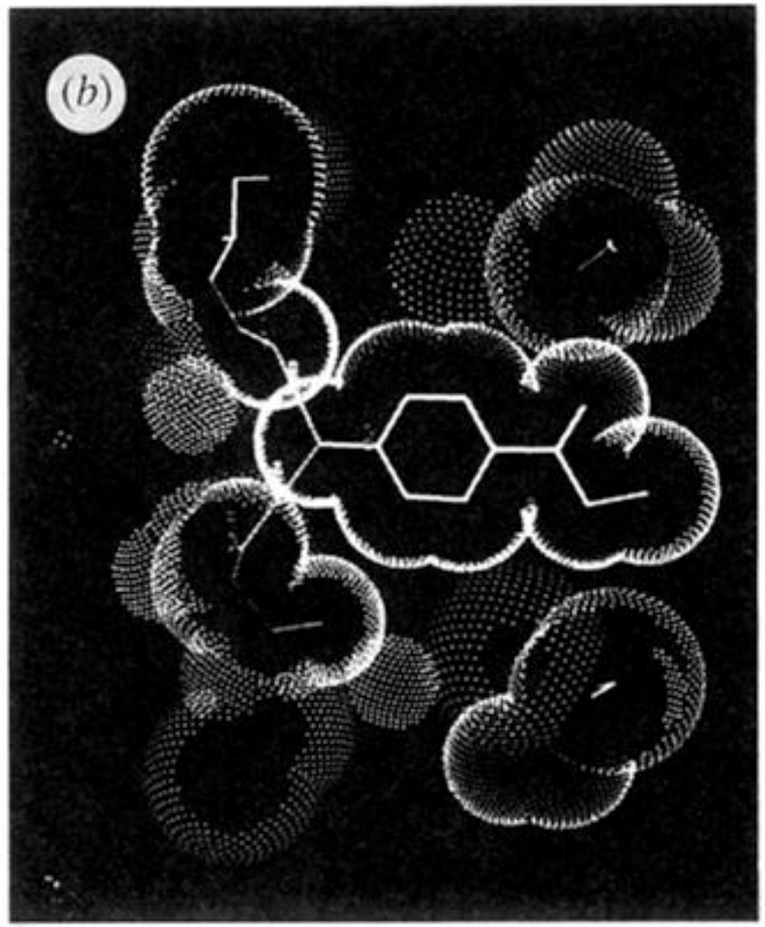


Figure 4. Dimers of (*a*) B9 asp, B27 glu, A8 his insulin and (*b*) B10 asp, B28 asp insulin, viewed down the local two-fold axis. The residues B9, B10, B12, B13, B27 and B28 are shaded in dark grey in both panels, A8 his is also shaded in (*a*) The pair of B13 glu immediately about the local two-fold axis seen in (*a*) are at H-bonding distance in the presence of the B9 asp mutation. In (*b*) the four carboxylic acids are separated and the closest interaction is within the monomer.





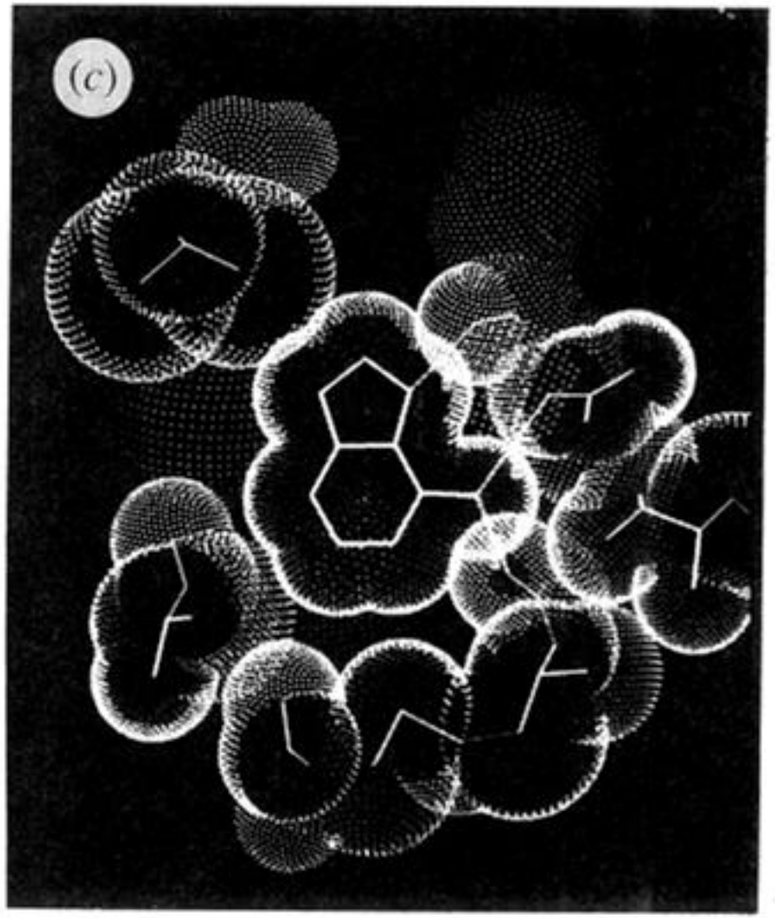


Figure 6. The environment at the phenol binding site in the R state insulin molecule; van der Waal radii are drawn as a dotted surface. The views differ slightly in order to reduce overlap of important structural features. (*a*) Phenol: its H-bonds to the peptide A6 CO and the A11 NH and the close approach of the B5 imidazole ring can be seen. (*b*) Methylparaben: there is an extra H-bond made through the carbonyl oxygen to a water molecule not shown. The methyl ester fills in extra space. (*c*) 7-OH-indole: the additional H-bond made by the indole-N to the A11 CO is made.