AN INVESTIGATION OF INSULIN STRUCTURE BY MODEL BUILDING TECHNIQUES*

by

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I. INTRODUCTION

Interest in the structure of insulin has increased recently as a result of the work of SANGER *et al.*[1,2,3] which, it is claimed, establishes the complete chemical structural formula of the unit of molecular weight *ca.* 6,000. The structural formula so proposed may be used to check the hypothesis that the α-helix of PAULING, COREY AND BRANSON[4], already well established as the basis of the structures of some synthetic polypeptides, is also a feature common to some fibrous and globular proteins. If this could be established it would give support to the view that this similarity of architecture extends also to the nucleoproteins.

This paper describes the first stage of an attempt to make such a check, by constructing a model of the molecule of insulin based on these hypotheses.

SANGER and his collaborators[1,2] have produced evidence that the insulin molecule is constructed from two types of peptide chain. One, the A chain, has the sequence of 21 residues, gly-ileu-val-glu(OH)-glu(NH_2)-cys-cys-ala-ser-val-cys-ser-leu-tyr-glu(NH_2)-leu-glu(OH)-asp(NH_2)-tyr-cys-asp(NH_2)§§. The B chain, which contains 30 residues, has the sequence phe-val-asp(NH_2)-glu(NH_2)-his-leu-cys-gly-ser-his-leu-val-glu(OH)-ala-leu-tyr-leu-val-cys-gly-glu(OH)-arg-gly-phe-phe-tyr-thre-pro-lys-ala. More recently SANGER and co-workers[3] have shown that sulphur bridges occur between A7 and B7, A20 and B19, and A6 and A11. Following HARFENIST AND CRAIG[5], SANGER interprets these results in terms of a unit of molecular weight *ca.* 6,000 composed of one A and one B chain. Neglecting initially all questions as

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§§ The abbreviations glu(OH), asp(OH) designate glutamic and aspartic acids respectively: glu(NH_2) and asp(NH_2) designate glutamine and asparagine.

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to the molecular weight of the insulin molecule, we set out to construct a model of the 6,000 unit. There have been previous attempts to construct models of the insulin molecule[6,7] but they have been based either on a 12,000 unit of four chains covalently linked through sulphur bonds or on an incorrect guess at the intrachain sulphur link. Our own initial investigations proved sufficiently encouraging for us to extend the model finally to the 12,000 dimer, but we have not attempted any investigation of the 36,000 or 48,000 unit although our model suggests ways in which such a molecule could be formed from the 12,000 unit.

II. DESCRIPTION OF THE TYPE OF MODEL BUILDING UNITS EMPLOYED

The type of model used has been developed in these laboratories by Professors PAULING AND COREY, and because of the advantages of strength and rigidity has proved extremely useful for the investigation of possible modes of folding of the peptide chain.

The models are of the non-spacefilling type constructed on a scale of 1" = 1Å, and are made of aluminium. The amide bonds are single planar units with provision for attaching two main chain bonds and two hydrogen bonds. These units correspond with the dimensions quoted by COREY AND PAULING[8], which are reproduced in Table I. Covalent bonds are steel dowel pins of the correct length and H bonds have a universal joint at the position corresponding to the oxygen atom so that the orientation of the receptor group is not restricted. Rigid attachment of the bonds is ensured by set screws. Two types of atom covered all requirements for the model.

TABLE I

DIMENSIONS USED FOR THE AMIDE GROUPS IN THE MODEL[8]

Dimension	*Value*
$C_\alpha - C$	1.53
$C = O$	1.23
$C - N$	1.32
$N - C_\alpha$	1.47
$N \cdots O$	2.72
$C_\alpha - C = O$	121°
$C_\alpha - C - N$	114°
$O = C - N$	125°
$C - N - C_\alpha$	120°
$C - N - H$	120°
$H - N - C_\alpha$	120°

A regular tetrahedral atom was used for tetrahedral carbon and nitrogen (as in $-NH_3^+$) and was sufficiently accurate to be used for oxygen (as in OH) and sulphur. Planar trigonal atoms with 120° bond angles were also used: these allowed construction of benzene rings and were used for both carbon and oxygen in carboxyl groups and carbon and nitrogen in the guanido group of arginine. The special problem of histidine was overcome by using the same trigonal atoms and bending the connecting bonds to the correct angle. For the dimensions of the main polypeptide chains overall errors not exceeding 2% may be expected. The appearance of models of this type should be made clear by Fig. 2, which will be described below.

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III. NOMENCLATURE

No systematic nomenclature for describing the various types of helical peptide chain structures has so far been published. PAULING has used a nomenclature in which the numbers of atoms in rings closed by H bonds from successive NH groups are given. For an α-helix this implies regarding the chain as commencing from the COO^- end. In this system an α-helix is described as 13, 13, 13, 13, 13—and a γ-helix as 16, 16, 16, 16, 16—. In general this nomenclature is satisfactory, but it would also be helpful to have some direct indication of which two residues are linked by H bonds. In an alternative nomenclature x–y represents an H bond from the CO of residue x to the NH of residue y. The numbering follows chemical convention, *i.e.*, starts from the NH_3^+ end of the chain. An α-helix thus becomes 1–5, 2–6, 3–7, 4–8, 5–9, 6–10, *etc.* Other nomenclature used is self-explanatory except perhaps in consideration of the 12,000 dimer where A, A′ and B, B′ are used to distinguish the pairs of A and B chains.

IV. THE A CHAIN

The α-helix of PAULING, COREY AND BRANSON[4] is well established as the basis of the structure of such synthetic polypeptides as poly-γ-methyl-L-glutamate. The evidence for its existence in the structures of proteins, and particularly of globular proteins, is less convincing. The particular case of the A chain of insulin offers a special difficulty since it is not possible to construct any intra-chain disulphide link on an undistorted α-helix. The α–β carbon-carbon bonds emerge at a constant angle from the axis of the helix and no two β carbon atoms are close enough to one another to be joined by a –S–S– link with the accepted configuration[9].

Attempts were made to devise other kinds of helix to get over this difficulty by causing the α–β carbon-carbon bonds to lie at different angles to the axis of the helix for different residues. In particular, helices were constructed in which the direction of the NH—O main chain H bonds was not uniform but in which sequences of NH—O vectors pointed towards either end of the helix. All such helices were unsatisfactory because of distorted bond angles, decreased van der Waals attraction as for the γ-helix or because side chain β-carbon atoms approached the main chain too closely.

A return was therefore made to consideration of the α-helix as the fundamental pattern with minor modifications designed to accommodate the A6–A11 disulphide link.

A model of a right handed α-helix was constructed in which the 6 residue was the D-half of *meso* cystine. It was, however, not possible to form a 6–11 intrachain disulphide link. There is moreover no conclusive evidence for the occurrence of meso-cystine in proteins.

A satisfactory solution to the problem of the intra-chain link which involves only L-cystine is a change of sense of the α-helix, from left handed to right handed at residue 9. This change of sense of the helix causes the α–β carbon-carbon bonds of the two halves of the L-cystine molecule to point towards each other so that a disulphide bond can be formed with an acceptable dihedral angle of *ca.* 90°. There are two ways of joining the two parts of the chain by H-bonds. One is more satis-

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factory than the other and this favoured structure involves the breakage of two main chain NH···O bonds. The NH group of residue 11 points towards the carbonyl oxygen of residue 6, but the N···O distance (3.5 A approx) is too great for the bond to be strong. The other carbonyl group, that of residue 8, can form an OH···O bond with the hydroxyl group of the adjacent serine residue 9, leaving only the NH group of residue 12 completely unbonded*. Using spacefilling models it can be shown that this NH group can coordinate a water molecule via an H-bond but this is not obvious using the non-spacefilling type of model. The structure imposes a bend of about 30° in the direction of the helix. Fig. 1 is a diagram of this system in which specific side chains are omitted except for the disulphide bond. The residue numbers increase from right to left so that residue 6 is the right and 11 the left half of the disulphide link. The α-carbon atom of residue 9, at the change of sense, appears directly above the S atom of residue 11.

Fig. 1. Diagram of proposed configuration of α-helix with change of sense to allow formation of an intrachain disulphide bond between residues equivalent to A6 and A11 in insulin. The point of change of sense is the α-carbon atom vertically above the left hand sulphur atom.

The hand of each part of the A chain α-helix is thus uniquely defined by the absolute configuration of the L-amino acids and by the short β-carbon to β-carbon distance in the cystine link. Residues A1–A9 form a L.H. helix and residues A9–A21 a R.H. helix. In the nomenclature of PAULING the structure can be described as 13, 13, 13, 13, 13, 13, 13, 13, 13, *a*, 16, 10, 13, 13, 13, 13, 13.

The first 13 refers to the H bond formed by the main chain NH of asparagine (A21), the 16 to the 3.5 A link and "*a*" represents the unbonded NH group of residue 12. In the alternative nomenclature the structure can be described as 1–5, 2–6, 3–7, 4–8, 5–9, 6–11 (this is the bond of length *ca.* 3.5 A), 7–10, 8 not bonded, 9–13, 10–14, 11–15, *etc.*, up to 17–21. The CO of 8 is bonded to the hydroxyl of serine (A9) and the NH of 12 is free. When such a model of the A chain is constructed various possibilities of side chain interaction appear. However, detailed consideration of these possibilities is out of place here, but it may be pointed out that the γ-carboxyl group of A4 can compensate the free NH_3^+ of the terminal glycine residue (A1).

* The less favourable configuration near the change of sense involves a hydrogen bond from CO6 to NH10. This leaves NH11, NH12, CO7 and CO8 unbonded. CO8 can form an OH···O bond with serine 9 but CO7 cannot be bonded by any side chain. NH12 is in such a position that it could form a bond to a water molecule but NH11 is completely masked by the β-carbon of residue 6. We have not investigated the packing between this form of A chain and either right or left handed B chains.

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V. THE B CHAIN AND THE A–B 6,000 UNIT

There is no *a priori* reason why the B chain cannot be considered as a simple α-helix distorted only by the presence of a proline residue near the carboxyl end. However, the two possibilities of L.H. and R.H. helix remain to be considered. In general an L-proline residue imposes a R.H. configuration on an α-helix since it is not sterically possible to fit it to a L.H. helix. However, in this case the sole proline residue occurs only two residues from the carboxyl end of the B chain and it is possible to construct a satisfactory model in which the B chain is an L.H. α-helix. ARNDT AND RILEY[7] claim to have shown from radical distribution curves on the isolated B chain that it is a R.H. helix. However, they also claim that intact insulin is a mixture of R.H. and L.H. helices. Acceptance of our proposed structure for the A chain and a R.H. α-helix for the B chain would imply that only 10% of the residues in insulin would be involved in a L.H. helix. This amount would almost certainly be undetected by the radial distribution technique. Moreover from the work of LINDERSTRØM-LANG AND SCHELLMAN[10] it would appear probable that the B chain as isolated by ARNDT AND RILEY was completely denatured.

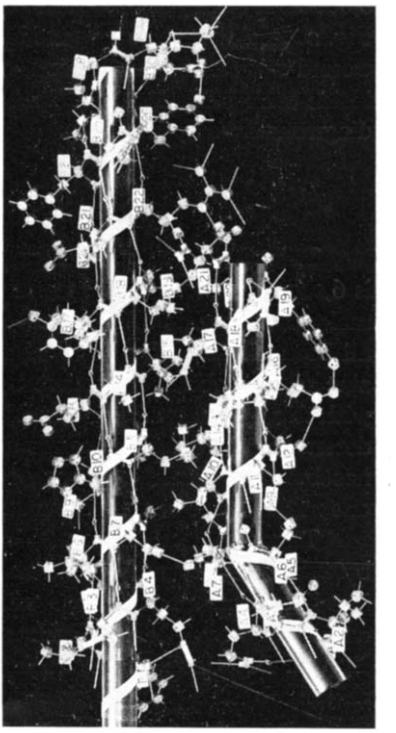

Fig. 2. Proposed model of the 6,000 molecular weight unit of insulin.

We have constructed models of the AB unit using the form of A chain described above with first a R.H. and then a L.H. α-helix for the B chain. For various reasons the first model appears to be preferable. For this preferred model the interchain disulphide bonds can be constructed with the correct dihedral angles and the A and B chains lie almost parallel over most of their length with a separation of 9.5 A between centers. The shorter bent portion of the A chain also lies in the same plane. The two sulphur links bridge opposite faces of the molecule and the interchain space is filled exclusively with hydrocarbon side-chains which can be packed closely without, however, approaching more closely than van der Waals contacts allow. The arginine residue (B22) can compensate the charge on the terminal carboxyl of the A chain (A21).

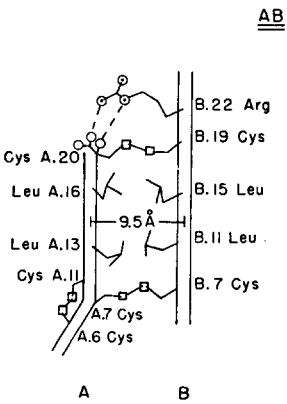

Fig. 3. Diagram illustrating main interactions between A and B chains in suggested structure.

It is still possible to construct an AB unit with sulphur bonds of the correct dihedral angle by use of the L.H. helix for the B chain. However, the A and B chains no longer lie parallel, the packing of side chains between the chains is not so extensive or regular and the arginine-asparagine (B22–A21) salt-link can no longer be formed.

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If the preliminary interpretation by Low[11] of the X-ray data on insulin sulphate is accepted, the molecule consists of a parallel array of rod-like structures. This provides further evidence against the B chain being an L.H. helix and supports our favoured model with the R.H. helix B chain. A photograph of a model of the AB 6,000 unit with the favoured R.H. helical configuration of the B chain is shown in Fig. 2. Residue numbers have been attached to the α-carbon atoms and a black core, wound with a white tape to indicate the track of the main chain, has been placed inside each helix. The principal interactions between the A and B chains are shown diagrammatically in Fig. 3.

VI. THE 12,000 UNIT

There has been some dispute whether the minimum molecular weight of insulin is 6,000 or 12,000[12,13]. It seems to us that these two values represent the minimum molecular weights of zinc-free- and zinc-insulin respectively. The 6,000 values have all been obtained by physical methods under conditions where the binding of the zinc to the protein may have been affected and amino acid analyses do not conflict with this minimum. The most definite evidence for the minimum of 12,000 is SCOTT's[14] determinations of metal contents of various forms of insulin (the work on zinc insulin has been confirmed by TANFORD AND EPSTEIN[15]) which demonstrate that there is one metal atom per 12,000 unit.

TANFORD AND EPSTEIN[15,16] have established that the zinc atom is coordinated by two of the four histidine residues of a 12,000 unit and have suggested that this is a link between two 6,000 units. If our assumption that the major part of the B chain is an undistorted R.H. α-helix is correct, then the two histidine residues at B5 and B10 on the same chain are too far apart to coordinate the same zinc atom and the bonding of the zinc must link two 6,000 units. Furthermore, it would be difficult to account for TANFORD AND EPSTEIN's result that the pK is different in zinc and zinc-free insulin for only two of the four histidine residues in a 12,000 unit if both B5 and B10 on every B chain were coordinated by zinc. There are thus three ways of incorporating the zinc atom; by bonding B5–B'5, B5–B'10 or B10–B'10.

In our judgment the most satisfactory structure is that in which B10 and B'10 are joined by the zinc and the two B chains are antiparallel. In this model the two histidine (B10 and B'10) and two glutamic acid residues (B13 and B'13) surround the zinc atom tetrahedrally so that the charges on the acid residues compensate that on the zinc. The distances assumed were Zn-N 2.0 A, Zn-O 2.7 A but the model is not sensitive to small changes in these dimensions. There is a twofold axis of symmetry through the zinc atom relating one 6,000 unit to the other, so that the pattern of interactions between the B chains is the same on either side of the zinc. Beyond the histidine-glutamic acid region there are van der Waals attractions between leucine residues B6 and B'17. Further out still it is possible for a salt link to be formed between a terminal amino group B1 and a γ-carboxyl group at B'21, provided that B1 is unwound from the α-helix by the breakage of one hydrogen bond. The NH group so freed can be hydrogen bonded by a glutamine side chain at B4 and the salt link is largely in a medium of low dielectric constant, being surrounded by two leucine (B6 and B'17) and three phenyl-alanine (B1, B'24 and B'25) side chains.

The separation of the two B chains bonded in this way is about 10.5 A and when

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the A and A′ chains are included the four chains lie very nearly in rectangular array. The density calculated from the model agrees very well with CROWFOOT's[17] observed value of 1.30. The rectangular array also fits LOW's X-ray data on insulin sulphate crystals, although it is not certain that the 12,000 unit is intact in this case. The only uncompensated charged side chains on the molecule are the two histidine (B5 and B′5) residues and the two glutamic residues on the A chains (A17 or A′17). The only significant possibility of AA′ interactions revealed by the model is a van der Waals interaction between tyrosine residues (A14–A′14).

VII. COMPARISON WITH CRYSTALLOGRAPHIC DATA

It is essential that any postulated structure for the molecule of insulin should fit into the unit cells determined for the air-dried protein by CROWFOOT[17] and for the wet protein by CROWFOOT AND RILEY[18]. CROWFOOT[19] has shown, by analysis of the Patterson diagrams for the two forms, that the broad features of the structure are the same in both. We may therefore use the cell dimensions for either form in any particular test of a proposed structure, taking whichever value is more difficult to explain. The symmetry of the structure is the same in both forms, R3 (C_3^4), and the dimensions of the rhombohedral and triply degenerate hexagonal cells are given in Table II.

TABLE II

UNIT CELL DIMENSIONS FOR WET AND DRY ZINC-INSULIN[17,18]

Dimension	*Wet*	*Dry*
a_R	49.4 A	44.4 A
α_R	114°16′	114°28′
a_H	83.0 A	74.8 A
C_H	34.0 A	30.9 A

If, as we assume, the B chain is an α-helix over most of its length, it is about 44 A long and cannot therefore run parallel or nearly parallel to C_H, whatever the form of the rest of the molecule.

The particular form of the 12,000 unit which we propose is approximately 30 A by 20 A by 20 A in size, with the carboxyl ends of the B chains projecting from this volume about 12 A in opposite directions parallel to the 30 A dimension. The unit cell contains threefold rotation and threefold screw axes running parallel to C_H and at *ca.* 25 A from one another. The 12,000 unit does not possess threefold symmetry and so cannot lie on a threefold rotation axis. It must not, therefore, be so placed that it overlaps any such axis. Similarly, no part of the unit which is more than 10 A in thickness may overlap a threefold screw axis.

These restrictions make it impossible that the major portions of the axes of the α-helices in the model we propose should lie nearly at right-angles to C_H. The only possible directions for these axes are at less than 50° but more than about 40° to the a_Ha_H plane, and packing considerations for an angle of about 45° from this plane indicate only one favorable position in which the molecule lies in the rhombus-shaped area between two rotation and two screw axes and the direction of the helix axes projects onto the a_Ha_H plane at about 45° to a_H. The packing of molecules so placed

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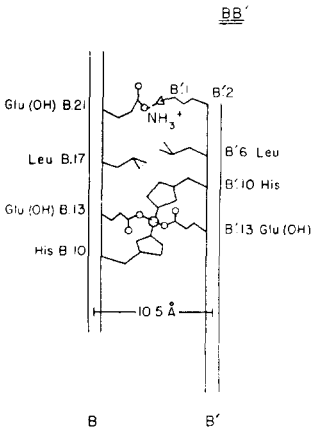

Fig. 4. Diagram illustrating main interactions between two B chains in suggested structure. Groups related by the twofold axis through the zinc atom are only drawn once except for the histidine and glutamic acid residues directly attached to zinc. The phenyl alanine side chains near B21 and B1 are omitted.

is shown in Fig. 5 and it can be seen that no two α-helix axes approach within 10 A of one another. There are several such possible structures differing by rotations of the molecule about the α-helix axis direction and no distinction has yet been made between them.

Tests have been made for the presence of α-helices in the three (symmetry related) orientations suggested by these packing arguments. PAULING AND COREY[20] have shown that the Patterson interatomic vector map of a structure containing an α-helix may be expected to show peaks at 5 A, 11.5 A and 16.5 A from the origin in the direction of the helix axis. They correlated these with peaks in the haemoglobin Patterson map. We are grateful to Mrs. DOROTHY CROWFOOT HODGKIN for supplying us with a list of intensities and a copy of her (unpublished) three dimensional Patterson map for wet zinc-insulin. This map shows density at 5 A, 11.5 A and 16.5 A from the origin in the required direction and so does not conflict with the structure proposed.

Comparison between the features of α-helix transforms for these orientations and the strong reflections led to inconclusive results, because of the large fraction of the volume of the reciprocal lattice occupied by strong regions of at least one of the symmetry related α-helix transforms.

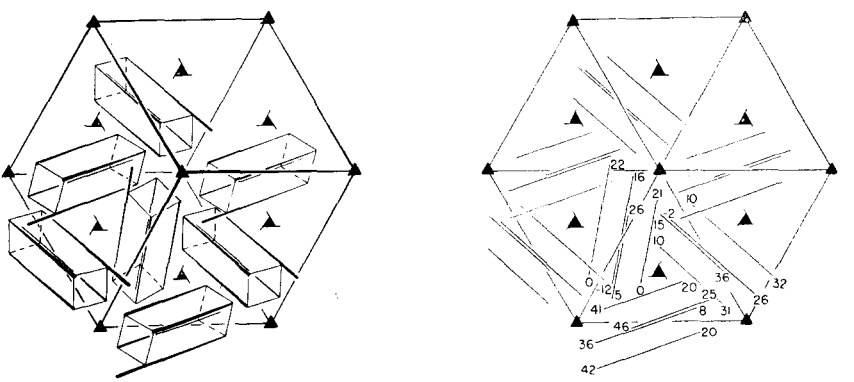

Fig. 5. Diagram of suggested arrangement of molecules in unit cell. Numbers at the ends of lines representing helix axes give heights in A units.

VIII. DISCUSSION

Undoubtedly this structure we propose for insulin is speculative but it should be realised that it is based on extremely few assumptions. We have assumed the correctness of the amino-acid sequences established by SANGER *et al.*; and of the α-helix configuration of the polypeptide chain proposed by PAULING AND COREY;

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that our suggestion for the structure of the A6–A11 sulphur bond is correct and that two 6,000 units are linked by a zinc atom complexed by the two B10 histidine residues. From this starting point we have made use of established principles of structural chemistry and the need for maximum charge neutralization and hydrogen bond formation. At each succeeding stage of the development of the structure this has led, in our judgment, to a general solution which was satisfactory structurally and made good chemical sense.

The principal conclusion to be drawn from this work is that it is possible to build a satisfactory model of a globular protein of known chemical constitution based substantially on the α-helix of PAULING, COREY AND BRANSON. This is of importance since although the α-helix has been generally accepted as the basic structure for fibrous proteins, doubts have been expressed regarding its applicability to globular proteins. The idea of a fundamental pattern of molecular architecture common to both fibrous and globular proteins was first advanced by ASTBURY and was extended by PAULING AND COREY to their α-helix structure.

The model also suggests ways in which this basic α-helix structure may be modified. The change of sense of the helix which we postulate for the A chain may have general significance. We have used it in order to bring about a closer approach of the β carbon atoms of the two halves of the cystine molecule, but the same principle could be used to bring other active side chains nearer together, or conversely by an opposite change of sense, to separate them more widely.

The serine side chain at A9 in the proposed structure can partially compensate for a broken main chain H-bond by forming an OH···O bond to a main chain carbonyl oxygen. This is a principle which is potentially capable of considerable extension. Other active side chains such as those of aspartic and glutamic acids and their amides could be utilized to stabilize changes of direction or sense of the α-helix.

The sequences deduced by SANGER *et al.* make it clear that the B30 COO^- group is near to the NH_3^+ of B29 (lysine). Similarly the NH_3^+ of A1 (glycine) is not far from the γ COO^- of the glutamic acid side chain at A·4. The proposed structure indicates an extension of this principle of neutralization of charges in that the COO^- of A21 is close to the positive arginine at B22 and the NH_3^+ at B1 can be linked to a COO^- at B'21 at the expense of one main chain H bond.

This last link is the only one of the four which is important in maintaining the configuration of the 12,000 unit. It is noteworthy that this link is shielded by three phenylalanine and two leucine groups from the dielectric effects of an aqueous medium[21].

These observations suggest that both intra- and inter-chain salt links occur in the structures of globular proteins although such structures may satisfy the electrostatic valence rule of PAULING[22] to some extent by the binding of ions from the solutions from which they are crystallized.

The proposed structure also provides a satisfactory explanation of some of the chemical properties of insulin, particularly its behavior on reduction. It is well known that reduction of one third of the sulphur bonds of insulin causes complete loss of biological activity[23,24]. Moreover it has been established that the activity cannot be restored by re-oxidation, rather any residual activity of insulin samples in which the extent of reduction is less than one third is decreased further when any attempt is made to re-oxidize the thiol groups to disulphide. Evidence obtained here by one

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of us (H.L.) suggests that the most readily reduced sulphur bond is the A6–A11 intrachain bond*. It can be readily understood why re-formation of this bond would be difficult, since the configuration of the chain at this point is dependent on this bond being intact. Once it is broken by reduction it is probable that the angle of bend in the A chain would increase to approximately 45° and the "long" 6–11 main chain H-bond could be formed at a more stable length. This would raise the energy of activation required for re-formation of the original disulphide bond. Re-oxidation would then take place preferentially with the formation of interchain disulphide bonds by some mechanism similar to the hypothesis of gel formation put forward by HUGGINS, TAPLEY AND JENSEN[25]. It is noteworthy that reduced insulin readily aggregates to high molecular weight products[26].

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SUMMARY

An investigation by model building techniques has shown that it is possible to build a model of the insulin molecule based on the α-helix structure and conforming to SANGER's chemical formulation. The model does not apparently conflict with chemical, physical or crystallographic evidence, but at present it can only be regarded as a possible trial structure which lacks any positive proof.

RÉSUMÉ

Les auteurs ont montré qu'il est possible de construire un modèle de la molécule d'insuline fondé sur la structure en hélice et conforme à la formule chimique de SANGER. Ce modèle est apparemment en accord avec les données chimiques, physiques ou cristallographiques, mais à l'heure actuelle on ne peut le considérer que comme une structure d'essai possible qui n'est étayé par aucune preuve positive.

ZUSAMMENFASSUNG

Die Möglichkeit, ein Modell des Insulinmoleküls auf Grund der α-Schraubenstruktur, und entsprechend der SANGERschen chemischen Formulierung, zu bauen, konnte durch eine Untersuchung an Hand von Modellbautechniken bewiesen werden. Das Modell steht zwar in keinem sichtlichen Widerspruch zu chemischen, physikalischen oder kristallographischen Befunden, kann jedoch gegenwärtig nur als eine mögliche Versuchsstruktur, ohne jegliche positive Beweise, angesehen werden.

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