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A Proposed Structure for Crystalline Zinc-Insulin

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A structure is proposed for crystalline zinc-insulin containing two atoms of zinc per unit cell. It is shown that the proposed structure is in agreement with the available chemical and crystallographic data for crystalline zinc-insulin.

A great deal of speculation has been devoted to the problem of constructing a satisfactory model of the unit cell of crystalline zinc-insulin. The specific information available for this purpose comprises: the complete amino acid sequence of the insulin monomer, the site of binding of the »structural» zinc ions, the amount of the »structural» zinc ions present in the cell, the molecular weight of 36×10^3 which indicates the presence of six monomers in the cell and finally the X-ray diffraction data.

A model based on the α-helix structure and Sanger's formula and constructed in accordance with model-building technique has been suggested by Lindley and Rollet [1]. The model proposed by these authors was constructed on the assumptions that the zinc was responsible for the dimerization and that the amount of zinc which belongs to the structure as an integral part was three atoms per unit cell and finally that the site of binding of the »structural» zinc atoms was the imidazole groups, each zinc atom being bound to two such groups.

It is now known, however, that zinc atoms are not necessary for the formation of the dimer as zinc-free insulin is able to dimerize. In fact the minimum molecular weight of zinc-free insulin at acid reaction has been shown [2] to be 12×10^3. In addition it should be pointed out that the binding of zinc to insulin is impossible at acid reaction where the behaviour of crystalline zinc-insulin is identical with that of zinc-free insulin in having a minimum molecular weight of 12×10^3 as also demonstrated by Gutfreund [3].

The problem about the amount of zinc which belongs to the structure as as integral part seems to be settled by the experiments performed by Schlichtkrull [4] and Cunningham [5]. By repeated crystallizations Schlichtkrull demonstrated that the minimum amount of zinc sufficient for crystallization was two atoms per unit cell. This result is in accordance with the work of Cunningham *et al.* who by equilibrium dialysis experiments showed that two atoms of zinc per unit cell were bound so firmly to insulin that they could not be dialyzed

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away. It is generally accepted that the building stone in the hexamer unit is the dimer, primarily because the monomer unit only seems to exist as such in aqueous solution under special conditions such as very dilute basic solutions (pH about 10). It can therefore be concluded that the unit cell of zinc-insulin consists of three dimers linked together by two zinc atoms and not by three zinc atoms as generally believed.

In a previous paper [6] it was demonstrated that the site of binding of the »structural» zinc atoms is the N-terminal amino group of phenylalanine and not the imidazole groups as suggested by Tanford and Epstein [7]. It has been shown previously too that the dimerization of insulin [8] probably proceeds by a disulfide interchange mechanism from which it could be concluded that the two monomers have an antiparallel arrangement in the dimer as shown schematically in Fig. 1.

The dimer thus has two carboxyl groups and two N-terminal amino groups in each end. The proposed structure then consists of three dimers placed in a parallel arrangement and linked together by two zinc atoms by means of coordinative bonds to the three available N-terminal amino groups of phenylalanine present in each end of the rod. The proposed structure is shown schematically in Fig. 2. As a consequence of the proposed structure it is seen that the addition of the calculated amount of zinc (two atoms per unit cell) to a solution of zinc-free insulin under certain circumstances should lead to the displacement of six hydrogen ions per unit of 36×10^3. In order to investigate this point titration curves for insulin with and without the presence of the calculated amount of zinc were determined. The results are shown in Fig. 3 An examination of Fig. 3 will show that in insulin containing two atoms of zinc per unit

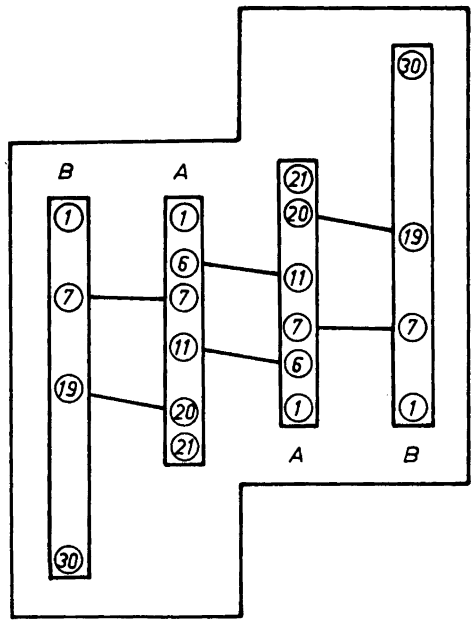

Fig. 1. The proposed model of the insulin dimer, showing the antiparallel arrangement of the monomers. The two longer rods represent the B-chains and the two shorter ones the A-chains. The numbers refer to the amino acids in the "Sanger-unit".

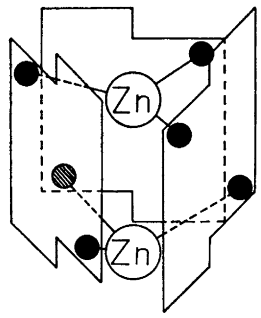

Fig. 2. The proposed model of crystalline zinc-insuiln. The three frames represents three dimers with structures similar to the one proposed proposed in Fig. 1. The black circles refer to the N-terminal amino groups of phenylalanine. The two water molecules probably attached to the two zinc atoms have not been drawn.

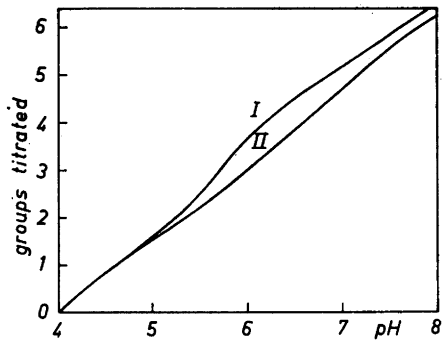

Fig. 3. Titration curves for insulin with and without the presence of two zinc atoms per unit of 36 × 10^3. The ordinate gives the number of groups titrated per unit of 6 000. Abscissa = pH. The titration were performed in 0.2 M KNO_3. I = titration curve for insulin in the presence of the calculated amount of zinc. II = titration curve for zincfree insulin.

of 36 × 10^3 the binding of six hydrogen ions has been shifted from the region about pH 7 towards more acid pH values. The result of this experiment therefore support the validity of the proposed structure.

The result of osmotic pressure measurements shows that crystalline zinc-insulin containing two atoms of zinc per unit of 36 × 10^3 is monodisperse in basic and neutral solution having a molecular weight of 36 × 10^3. This important fact is in accordance with the proposed model, since it is seen that zinc-insulin having this structure is not able to polymerize further by means of the two zinc ions. In the authors' opinion other possible structures would lead to compounds that are able to polymerize in solution depending on the insulin concentration. Such compounds would resemble zinc-free insulin in being heterodisperse in solution but as mentioned this is in diasgreement with experimental facts.

By isotope tracer experiments using ^{65}Zn Schlichtkrull [9] has shown that all the zinc ions present in the insulin crystal are exchanged within one hour, from which it can be concluded that the zinc ions are not captured within steric blockings. In the proposed model one zinc ion is placed in each end of the rod and it is therefore to be expected that zinc ions placed in this way would be easily exchangeable in agreement with the finding of Schlichtkrull.

CRYSTALLOGRAPHY

It is essential that any postulated structure of the hexamer unit of crystalline zinc insulin is in agreement with the available crystallographic data. Of special interest in this connection is the experiments performed by Low and Einstein [10] who by X-ray crystallographic studies showed that the insulin dimer basically has an invariant structure in acid solution, that is, the two insulin monomers in the dimer are related to each other by a twofold rotation axis. In Fig. 1 it is seen that the proposed structure of the dimer is in agree-

ment with this finding. A threefold rotation axis relating three dimer units has previously been found in rhombohedral zinc-insulin [11]. It appears therefore, as also pointed out by Low and Einstein, that the dimer as subunit of the hexamer may well have the point group 2. It is therefore probable that the hexamer has the point group 32 with each single molecule equivalent. If the hexamer unit has the point group 32 then the three twofold rotation axes relating the monomers to each other in the dimer subunit lie in a plane normal to the threefold rotation axis and at angles of 120° to each other. From Fig. 2 it is evident that the proposed structure fulfills all the necessary requirements for configurations belonging to point group 32.

Any postulated structure of the hexamer unit of crystalline zinc-insulin should be able to fit into the unit cell determined for the air-dried protein by Crowfoot [12]. The dimensions of the unit cell are given in Table 1. Calculations based upon the model proposed by Linderstrøm-Lang [13] for the monomer unit yields that the proposed model for the hexamer unit is 84 Å long and 30 Å broad. These dimensions correspond closely to the body diagonals in the unit cell determined by Crowfoot. It can therefore be concluded that the proposed structure is able to fit into the unit cell.

In aqueous solution the tails of the proposed model which are not stabilized by disulfide bonds, is supposed to be unfolded or in a state of equilibrium between the folded and the unfolded form in which the latter predominates. As a consequence of this the length of the proposed model would be considerably greater in aqueous solution than in the wet crystal. In the latter case an increase in the direction of the *a* axis should be seen, whereas the *c* axis should be the same as in the dry crystal since the width of the model is not affected by the unfolding of the tails. This is in exellent agreement with experimental facts since it has been shown, that on drying of the wet crystal the unit cell shrinks but only in the direction of the *a* axis, whereas the *c* axis remains practically constant [11].

Crystal growth studies of insulin crystals have been performed by Schlichtkrull [14]. In these investigations an anisotropic growth was demonstrated as only three faces of the crystal move whereas the other three faces are immobile. This indicates an up and down direction in the unit cell, that is, when the unit cell is turned upside down so as to invert the trigonal axis, it is impossible to obtain the original atomic configuration by rotating about this axis. This is in disagreement with the proposed model as shown in Fig. 2, where no up and down direction is present on account of the highly symmetric arrangement of the dimers.

It should be pointed out, however, that other parallel arrangements of the dimers in the hexamer are possible. In Fig. 4 A is schematically shown the arrangement of the dimers in the structure presented in Fig. 2. An alternate arrangement is shown in Fig. 4 B. As further illustrated in Fig. 5 the

Table 1. Lattice constants for air-dried insulin crystals determined by Crowfoot.

Rhombohedral cell	*Hexagonal cell*
a = 44.4 Å	a = 74.8 Å
α = 114°28'	c = 30.9 Å

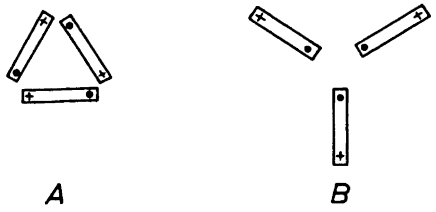

Fig. 4. Schematic illustration of two of the possible arrangements of the dimers. A rectangle illustrates a dimer; ● and + refer to the location of the N-terminal amino groups. ● amino group up and + amino group down. The zinc ions have not been drawn. 4 A refers to the structure proposed in Figs. 2 and 4 B to an alternate possible structure.

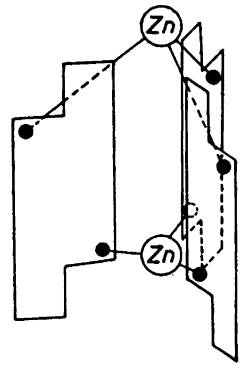

Fig. 5. A more detailed representation of the structure proposed in Fig. 4 B. The slight difference in the binding of the two zinc ions is apparent.

latter arrangement gives rise to a minor difference in the binding of the two zinc ions and consequently to an up and down direction in the unit cell. On account of the very slight difference in the ends of the rod the twofold rotation symmetry is lost.

Which one of these or other possible parallel arrangement of the dimers is the correct one it is at the moment impossible to say, but it seems that the main features of the proposed model are correct, since it is able to account for most of the available chemical and crystallographic data for crystalline zinc-insulin.

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