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The Binding of the "Structural" Zinc Ions in Crystalline Insulin

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The site of binding of zinc in crystalline insulin has been investigated by determinations of the molecular weight of insulin which has been substituted on the N-terminal amino groups by phenyl-*iso*cyanate and of insulin in which all carboxyl groups have been methylated. It is shown that the N-terminal amino groups are essential for the zinc-binding capacity of insulin whereas the carboxyl groups are without importance as regards the binding of zinc to insulin. In conclusion it is made probable that the zinc is attached to the N-terminal amino group of phenylalanine.

It has long been known that insulin can only be crystallized in combination with a trace of zinc ions. The minimum amount of zinc ions sufficient for crystallization, *i.e.* the amount of zinc ions which belong to the crystal structure as an integral part was originally believed to be three atoms of zinc per unit cell. By repeated crystallization Schlichtkrull [2], however, demonstrated that the minimum amount of zinc sufficient for crystallization was only two atoms per unit cell. This result is in accordance with the work of Cunningham *et al.*[3] who studied the zinc-insulin system by the equilibrium dialysis technique at pH 7.3—7.6 in barbiturate-acetate buffer. These investigations demonstrated that 0.6—0.8 gram atom zinc per 12 000 g of insulin were bound so tightly to insulin that they could not be dialyzed away. This amount of zinc corresponds very closely to the two atoms of zinc per unit cell which were found by Schlichtkrull to have a structural significance in the crystal structure of insulin. The site of binding of these two zinc ions has been investigated by Tanford and Epstein [4], who obtained the hydrogen ion titration curves of zinc-free insulin and crystalline zinc-insulin. The crystalline insulin used was claimed to contain three atoms of zinc per unit cell. By comparison of the two titration curves obtained and by calculations in which the three zinc atoms were treated alike as being identically bound to insulin the authors concluded that each zinc atom is bound to two imidazole groups. To the author this conclusion seems not the only possible one. The titration data of Tanford and Epstein does not exclude the possibility of the participation of α-amino groups in the binding of zinc to insulin as also pointed out by Gurd and Wilcox [5]. As mentioned Tanford and Epstein in their calculations treated the zinc atoms as

being bound to identical sites. In view of the findings of Schlichtkrull and Cunningham *et al.* it remains questionable if this assumption is correct. It would seem that one of the three atoms of zinc is bound by a weaker force than the other two.

In a previous publication [6] a difference in the behaviour of zinc-free insulin from that of crystalline zinc-insulin was demonstrated. It was shown that in the entire pH range investigated, pH 2.10—9.10, zinc-free insulin is inhomogeneous with respect to molecular weight, which depended upon pH and insulin concentration of the solution. This is in contrast to crystalline zinc-insulin which at neutral and basic pH is monodisperse in solution having a molecular weight of 36 000. It is therefore possible to obtain information about the site of binding of zinc to insulin by using zinc-free substituted insulin and measure the molecular weights by means of osmometry with and without the addition of the calculated amount of zinc sufficient for crystallization (2 atoms per unit cell).

The determinations of the molecular weights were performed as described in a previous publication [6]. In this investigation two substituted insulins * were used: One being substituted by phenyl*iso*cyanate, and one in which the carboxyl groups have been esterified by methanol. The insulin substituted by phenyl*iso*cyanate (*Iso*insulin) was prepared according to Hallas-Møller [7]. In this procedure two groups are substituted. By chromatography according to the method of Andersen [8] it was shown that the groups substituted were the N-terminal amino groups of phenylalanine and glycine. Phenylhydantoins of glycine and phenylalanine which were prepared by the method of Mouneyrat [9], were used as standards.

The methylinsulin was prepared by the method of Mommaerts and Neurath [10]. The methylinsulin was shown to be biologically inactive. Its methoxyl content was determined at 3.27 % (theoretical 3.05 %). It can therefore be concluded that all six available carboxyl groups have been esterified. Both the *iso*insulin and the methylinsulin were prepared free of zinc.

The determinations of the molecular weights of the substituted insulins with and without zinc were first performed at neutral pH. On account of the solubility properties of *iso*insulin, methylinsulin, and crystalline zinc-insulin it is unfortunately impossible to measure all three insulins at the same pH. Therefore the methylinsulin was measured at pH 6.7 and *iso*insulin and crystalline zinc-insulin at pH 7.4. The buffer used was barbiturate-acetate adjusted to an ionic strength of 0.06. The results of these measurements are recorded in Fig. 1. The curves I and II represent the results obtained with zinc-free *iso*insulin and methylinsulin. It is seen that the shape of these curves in identical with that obtained for zinc-free insulin which was recorded in a previous publication [6]. By addition of the calculated amount of zinc (the zinc was added as the chloride) however, the curve representing the results obtained for zinc-free methylinsulin is transformed into curve III, which represents a monodisperse compound having a molecular weight of 36×10^3. By addition of the calculated amount of zinc to *iso*insulin it is seen that no effect could be demonstrated thus indicating that *iso*insulin is unable to bind zinc whereas the opposite is true of methylinsulin. Crystalline zinc-insulin containing two zinc atoms per unit cell is used as reference. The results obtained with this sample

* The substituted insulin was kindly prepared by "Novo Terapeutisk Laboratorium".

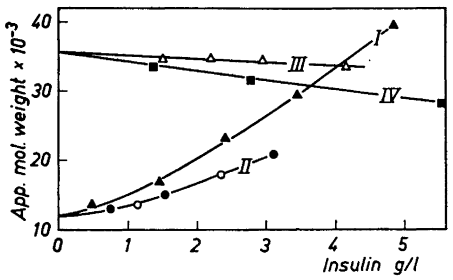

Fig. 1. Molecular weight determinations on *iso*insulin, methylinsulin and crystalline zinc-insulin. Ordinate: Apparent molecular weight; Abscissa: Concentration in g/l. I and III: Methylinsulin, ▲ without Zn^{++}, △ with Zn^{++}, pH = 6.7; II: *Iso*insulin, ● without Zn^{++}, ○ with Zn^{++}, pH = 7.4; IV: Crystalline zinc-insulin, pH = 7.4. All measurements performed in barbiturate-acetate buffer. I = 0.06 and temperature 20°C.

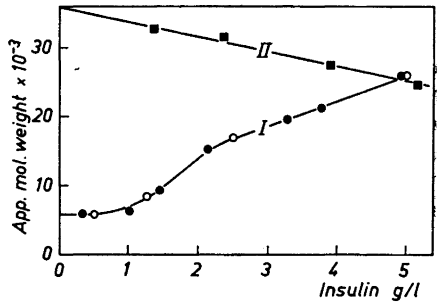

Fig. 2. Molecular weight determinations on *iso*insulin and crystalline zinc-insulin. I: *Iso*insulin, ● without Zn^{++}, ○ with Zn^{++}; II: Crystalline zinc-insulin. All measurements performed in barbiturate buffer. pH = 8.6, I = 0.2 and temperature 20°C.

are represented by curve IV, which indicates a monodisperse compound with a molecular weight of 36 × 10^3. The difference in the slopes of curve III and IV may be due to the difference in charges on methylinsulin and crystalline zinc-insulin.

In order to examine the behaviour of *iso*insulin more closely measurements were performed at pH = 8.6 in barbiturate buffer adjusted to the ionic strength 0.2 by addition of KCl. Crystalline zinc-insulin containing two zinc atoms per unit cell was again used as reference. The results are recorded in Fig. 2. It is clearly demonstrated that addition of the calculated amount of zinc has no effect on the behaviour of *iso*insulin.

From the experiments reported here one can safely conclude that the N-terminal amino groups of insulin are essential for the zinc-binding capacity of insulin, whereas the carboxyl groups seem to be without importance with respect to the binding of zinc to insulin. In insulin, however, there are two N-terminal amino groups, one from glycine and one from phenylalanine and the question still to be solved is the problem whether the zinc is bound to both amino groups or only to one of them. Support for the latter view comes from the experiments performed by Christensen [11] and especially from the work of Andersen [8]. Using the Edman procedure Christensen demonstrated that the N-terminal amino group of phenylalanine in crystalline zinc-insulin reacted to a much lesser extent than did the N-terminal amino group of glycine. By substitution of crystalline zinc-insulin with phenyl*iso*cyanate Andersen demonstrated that the first group to be substituted by this reagent was the N-terminal amino group of glycine. The N-terminal amino group of phenylalanine reacted more sluggishly with a considerably lower reaction rate. By column chromatography Andersen was able to separate two reaction products;

a mono-derivative which was substituted at the N-terminal amino group of glycine and a di-derivative which was substituted at both the available N-terminal amino groups. By sedimentation experiments performed with crystalline zinc-insulin and the mono- and di-derivatives of the substituted insulin Andersen [12] showed that crystalline zinc-insulin and the mono-derivative behaved identically in the ultracentrifuge as monodisperse compounds having a molecular weight of 36×10^3. The behaviour of the di-derivative in the ultracentrifuge was quite different from the behaviour of crystalline zinc-insulin and the mono-derivative. The di-derivative showed an inhomogeneous pattern and, under the experimental circumstances chosen, had a higher molecular weight than the crystalline zinc-insulin. This result is in complete agreement with the osmotic pressure data recorded here. Andersen interpreted his results in terms of interaction of non-polar groups, but in view of the findings herein presented a much more plausible explanation is possible. The explanation simply implies that the "structural" zinc is attached to the N-terminal amino group of phenylalanine. By assuming this it is readily understandable that the N-terminal amino group of glycine is the first to be substituted. Furthermore it is to be expected that substitution of this amino group will have no influence on the structure of the zinc-insulin complex, *i.e.* the substituted insulin should be monodisperse having a molecular weight of 36×10^3 in complete agreement with the findings of Andersen. By substitution also on the N-terminal amino group of phenylalanine, however, the structure of the insulin would be expected to deteriorate, since zinc is essential for the structure of the hexamer unit (mol.wt. 36×10^3). The behaviour of the di-substituted insulin should therefore be identical with the behaviour of zinc-free insulin in being polydisperse and having a molecular weight depending upon the insulin concentration and pH of the solution. This prediction is in complete accordance with the results obtained in this investigation and with the results obtained by Andersen. The above mentioned experiments therefore strongly support the view that in crystalline zinc-insulin the "structural" zinc is attached to the N-terminal amino group of phenylalanine.

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