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The Dimerization of Insulin

KJELD MARCKER and JOHN GRAAE

Universitetets fysisk-kemiske Institut and Universitetets hygiejniske Institut, Copenhagen, Denmark

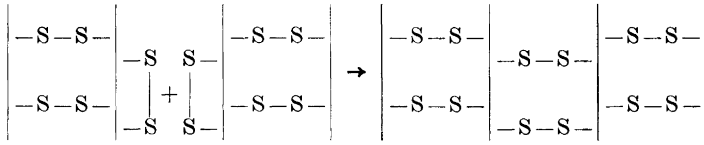
The dimerization of insulin has been investigated by determinations of the molecular weight of insulin which has been treated with silver ions, and of insulin treated with cyanide. It is shown that silver ions prevent the formation of the dimer and that the addition of cyanide results in a reversal of the dimerization. In conclusion it is proposed that the dimerization of insulin proceeds by a disulfide interchange mechanism. The silver-insulin compound has been shown to be biologically active with a slightly prolonged effect.

It has long been known that insulin is polymerized in aqueous solution. From numerous investigations it is known that the polymerization depends upon the presence of metal ions, especially zinc, in the solution. Crystalline zinc-insulin containing two atoms of zinc per unit cell has been shown to be monodisperse in solution at basic and neutral pH having a molecular weight of 36 000 [1] which corresponds to six monomer units. It is generally believed that the structure of the zinc containing unit cell (*M.* 36 000) consists of three molecules having a molecular weight of 12 000 combined by means of the two zinc atoms. In a previous publication [1] it has been shown that the site of binding of the zinc is the N-terminal amino groups of insulin.

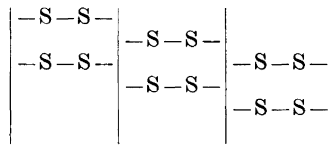
The behaviour of zinc-free insulin, however, is quite different. In the entire pH range zinc-free insulin is inhomogeneous with respect to molecular weight which depends on pH and insulin concentration [2]. While the association of insulin by means of zinc seems fairly well understood, nothing definite is known concerning the association of zinc-free insulin. It has been suggested that the latter may proceed by interactions of non-polar groups [3]. Anyhow it seems that the association monomer to dimer proceeds by a reaction which is different from the one leading to association of dimers to higher polymers, because the dimer is a rather stable unit. In fact it was for a long time believed that 12 000 was the minimum molecular weight of insulin. It should also be pointed out that the dimer is the building stone in the zinc-containing unit cell. It must be stressed, however, that the suggestions [4,5] hitherto reported concerning the difference between dimerization and subsequent polymerization have been of a purely speculative nature, but the results of a previous

experimental investigation [2] seemed to indicate that the difference actually exists.

In 1956 Fredericq [5] proposed that the dimerization proceeds by a disulfide interchange mechanism as shown in the following scheme:



This suggestion was rejected when Sanger [5] pointed out that this structure by hydrolysis would lead to the formation of peptides other than those actually obtained by him. It was also stated that the outer disulfide bridge has a stability similar to the stability of the two other disulfide bridges. Sangers arguments, however, only hold if it is assumed that the two monomers have a parallel arrangement in the dimer, but assuming an antiparallel arrangement, having the following schematic formula:



one would get exactly the same peptides as were isolated by Sanger. Concerning the question about the stability of the disulfide bridges Lindsey [6] has later shown that the outer disulfide bridge actually is more labile than the other two.

It is now generally accepted that sulfhydryl-disulfide reactions proceed by a cleavage of the disulfide bond induced by the mercaptide ion [7,8]:

$$R{-}S{-}S{-}R + R'S^- \rightarrow R{-}S{-}S{-}R' + RS^- \quad (1)$$

$$R{-}S{-}S{-}R' + R'S^- \rightarrow R'{-}S{-}S{-}R' + RS^- \quad (2)$$

It is to be expected that interchange reactions of this type would proceed at a more rapid rate as the pH and thus the mercaptide ion concentration is increased. In the case of compounds which contain no sulfhydryl group, the disulfide interchange reaction in neutral or basic solution appears to proceed by a similar mechanism [7,8], the initiating sulfhydryl group being produced by the hydrolysis of the disulfide bond:

$$R{-}S{-}S{-}R + H_2O \rightarrow RSOH + RSH \quad (3)$$

Most observations of sulfhydryl-disulfide interchange and disulfide interchange in proteins have been concerned with phenomena accompanying protein denaturation, chiefly those of aggregation, but these reactions have also been shown to participate in reversible protein association in the absence of denaturing agents, *e.g.* the dimerization of bovine plasma albumin [9] and the asso-

ciation of soluble feather keratin [10]. These associations have been shown to be freely reversible by such relatively mild manipulations as dilutions or changes in pH, which have the same effect upon insulin association [2]. The dimerization of bovine plasma albumin can be reversed or completely inhibited by addition of mercury chloride. In the experiments reported here silver nitrate is employed instead of mercury chloride since mercury ions have been shown to be involved in the formation of protein dimers under certain conditions [11].

The insulin preparations used were zinc-free insulin and crystalline zinc-insulin containing two atoms of zinc per unit cell. Determinations of the molecular weight were performed by means of the inverted microosmometer described by Christiansen and Jensen [12].

Zinc-free insulin was dissolved in 0.2 M KNO_3 at pH 8.6 to give a concentration of 0.1 %. Under these conditions insulin consists mainly of the monomer [2]. The calculated amount of $AgNO_3$ (one mole per monomer) was added and the solution was then acidified to pH 2. At this pH insulin mainly is present as the dimer [2]. Finally the molecular weight was determined.

From the results recorded in Table 1 it is clearly seen that the presence of silver ions effectively prevents the formation of the dimer. It is also seen that the addition of silver ions results in a breakdown of the hexamer unit into monomers. The solubility of the silver insulin differs from that of insulin itself. The range of the isoelectric precipitation of the silver-insulin is extended towards the acid region to about pH 2.

Treatment of zinc-free insulin with cyanide was also tried. Cyanide is known to cleave disulfides in the following manner [7,8]:

$$R-S-S-R' + CN^- \rightarrow RSCN + R'S^- \quad (4)$$

It was therefore expected that treatment of insulin with cyanide would lead to a splitting of the dimer into monomers. The result of this experiment is recorded in Table 2 from which it can be concluded that treatment of insulin with cyanide results in a reversal of the dimerization.

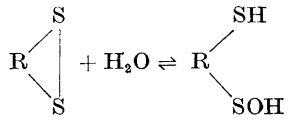
The simplest explanation of the results reported in this paper is that the dimerization of insulin proceeds by a disulfide interchange mechanism. These reactions proceed by the intermediate formation of a mercaptide ion by a hydrolytic cleavage of the disulfide:

Table 1. Apparent molecular weights of different insulin preparations treated as described in the text. All preparations dissolved at pH 8.6.

	Treatment	App.mol.wt.	pH
Zinc free insulin	Ag^+ added at pH 8.6	5.9×10^3	2
» » »	Ag^+ added at pH 2	9.3×10^3	2
» » »	No addition of Ag^+	10.5×10^3	2
Crystalline zinc insulin	Ag^+ added at pH 7	5.7×10^3	7

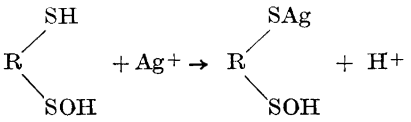
Table 2. Apparent molecular weight of insulin incubated with cyanide under a nitrogen atmosphere. 0.0110 mmole insulin (M 6 000) treated with 0.0120 mmole NaCN. pH = 7.5. 0.1 M barbiturate buffer.

Hours of incubation	0	20	40
App. mol. wt.	15×10^3	6.8×10^3	6.5×10^3



(5)

and the observed inhibition of the dimerization process by silver ions is then readily explained by the following reactions:



(6)

The reversal in the dimerization observed by cyanide treatment supports this point of view, since cyanide under the experimental conditions chosen has been shown to be highly specific for disulfides [7,8]. It is interesting to note in this connection that oxytocin [13] which has a disulfide bridge with the same configuration as the one present in the A chain in insulin has been shown to dimerize by the same mechanism as here proposed for insulin.

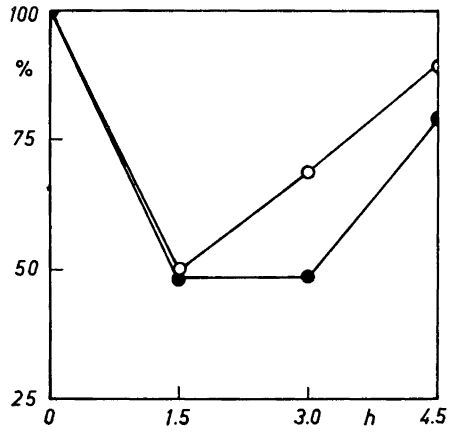


Fig. 1. The action of zinc-free insulin (—○—○—) and silver insulin (—●—●—) in fasting rabbits. Ordinate: Bloodsugar in per cent of initial bloodsugar. Abscissa: Hours after injection. Dose: 0.75 units per rabbit. Cross-over assay on 9 rabbits.

The biological activity of the silver insulin has been tested on rabbits. The result is shown in Fig. 1. Silver insulin is not able to polymerize but exists only as the monomer. Fig. 1 clearly illustrates that this unit is biologically active. The slightly prolonged effect might be due to an inhibition of one of the insulinases present in tissue.

The mentioned dimerization of oxytocin leads to inactivation, and if it is assumed that the dimerization of insulin has the same effect, the dimer would serve as a physiological storage product. Reactivation could then easily be accomplished by a simple dilution by which the monomer is produced. Whether or not the proposed disulfide interchange mechanism plays a role in the physiological action of insulin remains to be investigated, but it is of special interest in this connection that Klotz [14] has pointed out that such a mechanism could furnish a means of electron transport in oxidation-reduction involving sulfhydryl enzymes.

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REFERENCES

1. Marcker, K. *Acta Chem. Scand.* **14** (1960) 2071.
2. Marcker, K. *Acta Chem. Scand.* **14** (1960) 194.
3. Oncley, J. L., Ellenbogen, E., Gitlin, G. and Gurd, R. R. N. *J. Phys. Chem.* **56** (1952) 85.
4. Lindley, H. and Rollett, J. S. *Biochim. et Biophys. Acta* **18** (1955) 183.
5. Fredericq, E. *Ciba Foundation Colloquia on Endocrinology* **9** (1956) 119.
6. Lindsey, H. *J. Am. Chem. Soc.* **77** (1955) 4927.
7. Foss, O. *Kgl. Norske Videnskab. Selskab, Skrifter* **18** (1945) No. 2.
8. Parker, A. J. and Kharasch, N. *Chem. Revs.* **59** (1959) 583.
9. Bro, P., Singer, S. J. and Sturtevant, J. M. *J. Am. Chem. Soc.* **80** (1958) 389.
10. Woodin, A. M. *Biochem. J.* **57** (1954) 99.
11. Hughes, W. L. J. *J. Am. Chem. Soc.* **69** (1947) 1836.
12. Christiansen, J. A. and Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 1247.
13. Ressler, C. *Science* **128** (1958) 1281.
14. Klotz, J. M., Ayers, J., Ho, J. Y. C., Horowitz, M. G. and Heiney, R. E. *J. Am. Chem. Soc.* **80** (1958) 2132.

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