INSULIN DISSOCIATION AT ALKALINE pH

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1. Introduction

Insulin shows a typical quaternary structure strongly influenced by pH, metal ions concentration and other factors [1–3]. The equilibria between monomer and polymers have been studied mainly by sedimentation analysis under various conditions [4].

In a previous work we have demonstrated that insulin, left for suitable time in alkaline solution at a pH higher than 12, yields persulfide groups by a mechanism of α–β elimination of the cystinyl sulfur [5]. In these conditions dehydroalanine residues are produced. They could be the elective site for a possible hydrolytic scission of protein chains, with formation of pyruvoyl-peptide derivatives [6–7].

This paper reports our studies on the effect of alkaline treatment on the quaternary structure of insulin, investigated by gel filtration on Sephadex G-50, in conditions where α–β elimination reaction is minimal or absent.

2. Experimental

Beef crystalline zinc-insulin was a generous gift of Wellcome Italia; it had a Zn content of 0.38%, 6% of moisture and an activity of 24 UI per mg. Ribonuclease was purchased from Fluka (Buchs); Sephadex G-50 fine and blue dextran were obtained from Pharmacia (Uppsala). The gel was swelled, equilibrated with 0.01 N NaOH at room temp. and packed into a 0.8 × 80 cm column. This kind of column does not allow to separate peptides arising from cleavage of insulin monomer, but a complete separation between dimer and monomer is obtained. The gel chromatographic analyses were performed at room temp. and at a constant elution rate of 10 ml per hr with peristaltic pump. Blue dextran (0.5 mg) was used as marker dye for void volume determination and ribonuclease as standard protein for a rough evaluation of molecular weights.

Insulin was dissolved, at a concentration of 3.3 mg per ml, in 0.01 N NaOH or in the same solution containing 6 M urea. Immediately or after 12–24 hr the solutions were submitted to chromatographic separation in 0.01 N NaOH.

In a second experiment, insulin, at the same concentration, was pretreated with 0.05 N NaOH at room temperature: in this condition α–β elimination reaction occurs at high rate. After different incubation times the excess of NaOH was exactly titrated by addition of a suitable amount of HCl to obtain a final concentration of 0.01 N NaOH. This procedure allows to stop completely the α–β elimination reaction, but the pH is high enough to ensure full stability of persulfide groups formed [5]. Therefore the chromatographic separation also has been performed using 0.01 N NaOH as solvent.

3. Results and discussion

Fig. 1 shows the influence of 6 M urea on insulin in 0.01 N NaOH. The dotted line represents the elution pattern of untreated insulin, whose peak corresponds roughly to the elution peak of ribonuclease. In these conditions the hexameric form of insulin changes rapidly into the dimeric one, which is

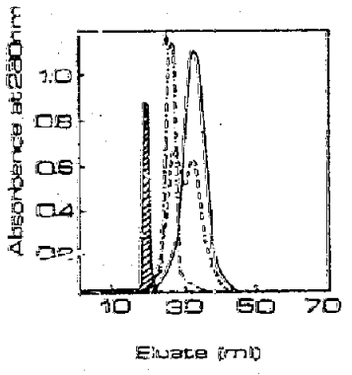

Fig. 1. Effect of urea treatment on the chromatographic behaviour of Zn-insulin (5 mg) in G-50 fine Sephadex column; elution solvent 0.01 N NaOH. Untreated: (------); after 12 hr 6 M urea treatment (----); after 24 hr 6 M urea treatment (——); shaded peak: blue dextran. The arrow shows the elution volume of ribonuclease.

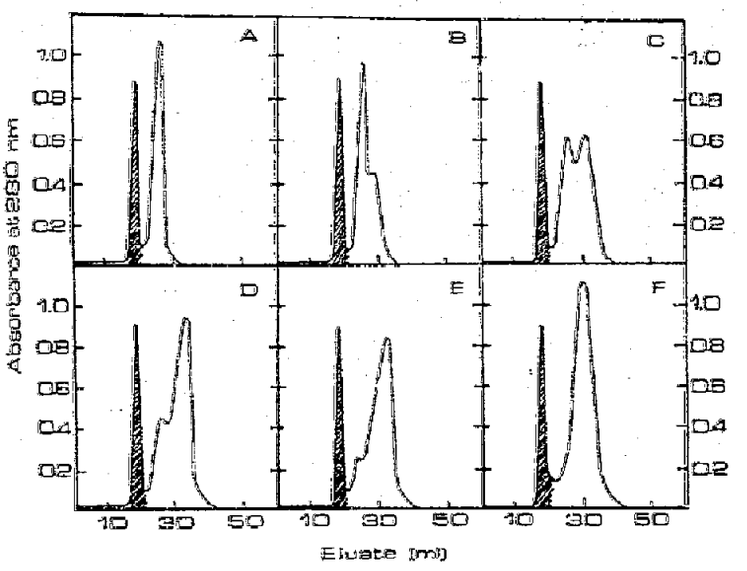

Fig. 2. Effect of NaOH treatment on the chromatographic behaviour of Zn-insulin (5 mg) in G-50 fine Sephadex column; elution solvent 0.01 N NaOH. A) Untreated; treated for various times with 0.05 N NaOH. B) 15 min; C) 30 min; D) 1 hr; E) 3 hr; F) 6 hr. The shaded peak represents the elution volume of blue dextran.

completely stable. When it is dissolved in urea and immediately applied on the Sephadex column, the eluted peak is completely superimposable to that of untreated insulin. After 12 hr in 6 M urea the peak splits into two components; it is obvious to suppose an equilibrium between dimer and monomer. If the treatment is prolonged for 24 hr the transformation is complete and only the monomeric form is present. A control performed with the protein left for 24 hr in 0.01 N NaOH, without urea, shows no formation of monomer at all.

A quite similar behaviour is observed when insulin is treated with 0.05 N NaOH without urea. Fig. 2 shows the elution patterns of insulin at various incubation times, after solutions have been treated as described in the experimental section; chromatography is always performed in 0.01 N NaOH. Yet 15 min in 0.05 N NaOH are enough to get the formation, in little amount, of the lighter component, which has a molecular weight similar, if not identical, to that of the monomer obtained by urea dissociation (fig. 2,b). The time course of this transformation is easily followed on fig. 2 (c, d, e, f). After 6 hr no more dimer is present, as could be expected from the data previously reported [5]. In fact the extinction at 335 nm of the incubation mixture, due to persulfide formation, reaches its maximum after 2 hr of treatment in 0.05 N NaOH. Of course both phenomena do not follow the same time course, although they are interconnected. Whereas the persulfide formation shows an initial lag period, the transformation into monomer due to alkalinity seems to begin at once.

This fact could be explained easily if we consider that the appearance of persulfide groups, induced by NaOH action, is only possible when a suitable amount of monomer is present, which enables disulfide bridges to be well exposed to the action of solvent. The accessibility of disulfide bonds in function of polymerization degree is not easy to follow, but in this context we could affirm that in dimeric, and, perhaps, higher forms of insulin the disulfide bridges are to some extent protected against the solvent action. This interpretation is supported by the fact that in 0.05 N NaOH we observe a more rapid appearance of persulfide groups if 6 M urea is present, owing to a concomitant action of both dissociating agents.

The complete disappearance of the dimeric form is observed, by gel filtration, after 6 hr. On the other side we were not able to discriminate between monomer and polypeptides of minor molecular weight, which could arise from A or B chains. In fact it can not be excluded that, also in these experimental conditions, small amounts of peptides are produced by the alkaline scission on A or B chains, at the level of dehydroalanine residues, as has been reported for the

insulin treated with 0.2 N NaOH [6]. However this problem is not essential in this context. Further work is in progress to elucidate this fact, in connection with the more general problem of the α–β elimination reaction mechanism in insulin.

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References

[1] E. Fredericq, Arch. Intern. Physiol. 74 (1956) 132.
[2] C. Tanford and J. Epstein, J. Am. Chem. Soc. 76 (1955) 2170.
[3] L.W. Cunningham, R.L. Fischer and C.S. Vestling, J. Am. Chem. Soc. 77 (1955) 5703.
[4] E. Fredericq, Arch. Biochem. Biophys. 65 (1956) 218.
[5] D. Cavallini, G. Federici, E. Barboni and M. Marucci, FEBS Letters 10 (1970) 125.
[6] G. Federici, F. Bossa, E. Barboni and D. Cavallini, Atti Simposio Internazionale Technicon, Roma, 1971.
[7] M. Sokolovsky and A. Patchornik, J. Am. Chem. Soc. 86 (1964) 1859.