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The self-association of zinc-free human insulin and insulin analogue B13-glutamine *

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The self-association of Zn-free human insulin, Zn-free insulin analogue B13-glutamine, 2-Zn insulin and cobalt(III) human insulin in the millimolar concentration range has been investigated by measuring the osmotic pressure at pH 7.5 in 0.05 M NaCl, 25 °C. The pH dependence of association has been measured in the pH range 6.8–9. For all insulins, except Zn-free human insulin, the major association state has been found to be the hexamer. Maximal association of hexamer has been observed for Zn-free human insulin at high concentration (2–7 mM) and physiological pH. At concentrations less than 1 mM and pH greater than 7.0, dissociation to a lower state than the hexamer is found. The conclusion has been drawn that, in the absence of metal ions, human insulin and insulin analogue B13-glutamine associate to the hexamer in the physiological pH range at concentrations in the millimolar range.

1. Introduction

The self-association of insulin has been studied extensively [1–7]. This property of insulin is not only of scientific interest, as it has been shown that there is a correlation between the association state and the subcutaneous absorption rate of insulin and insulin analogues [8].

In the crystal structure and under physiological conditions at millimolar concentrations, the 2-Zn insulin is found as a hexamer [7]. By dilution down to physiological concentration (nanomolar range), 2-Zn and Zn-free insulin dissociate to the dimer and monomer, respectively [1].

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The self-association of Zn-free insulin has been studied predominantly using equilibrium ultracentrifugation [1–5].

A model of association involving monomers, dimers, and hexamers as well as higher aggregates has been proposed by Pekar and Frank [1]. Subsequently a linear association model describing the association at pH 7.0 was proposed by Jeffrey and co-workers [3–5].

In the theory of equilibrium ultracentrifugation, the data may be considered as equilibrium values but in real cases nonideality must be taken into account [9]. In ultracentrifugation studies of insulin association, pressure effects on the distribution of the different association states have been neglected [1,3,6]. In the present study, the concentration and pH dependence of insulin association is described by using a real equilibrium method, based on the osmotic pressure, which in the actual molecular weight range can be used in

the millimolar range of concentration. The elucidation of the pH and concentration dependence in the millimolar range is important in understanding the association of Zn-free insulin. The influence of B13-glutamates on the association [7] has been studied by comparing the association of insulin analogue B13-glutamine with that of human insulin.

2. Materials and methods

2.1. Proteins

Metal-free human insulin with less than 0.002 Zn^{2+} per hexamer (protein purity > 99%, salt-free) was used. Insulin analogue B13-glutamine had the same specifications as for human insulin.

The cobalt (III) insulin hexamer was prepared following the procedure of Dunn et al. [10]. Protein concentration was determined spectrophotometrically using values for the absorbance at 276 nm of 1.05 for 1 mg/ml insulin and 1.34 for cobalt (III) insulin hexamer.

Ovalbumin was obtained from Sigma (grade V) and the value $E_{1\%}^{280\,nm} = 7.12$ [11] was used.

2.2. Osmometry

A Knauer type 1.00 membrane osmometer fitted with a water thermostated shell to permit measurements below 30 °C was employed. A Schleicher and Schuell AC 61 membrane (retention 10 kDa) was used. In the case of insulin, an exclusion limit of less than 6 kDa has been determined with monomeric insulin analogues [8]. Calibration is performed with external water pressure. In order to check the calibration, the molecular mass of ovalbumin was determined. A value of 43.5 ± 0.8 kDa was obtained. This is in accordance with the value of 43.5 kDa reported by McCarty and Adams [11].

The solvent used was 50 mM NaCl. This ionic strength has been found to be high enough to avoid the influence of the Donnan term on the osmotic pressure, and sufficiently low to prevent the precipitation of insulin as sodium insulinate crystals.

Buffer was not used, since insulin itself at millimolar concentrations has sufficient buffering capacity to maintain the pH during the measurements. The duration of one measurement period was approx. 1 h.

3. Results

The equation describing the concentration dependence of the osmotic pressure is:

$$\frac{\pi}{c} = \frac{1}{\overline{M}_n} RT\left(1 + \Gamma_2 c + \Gamma_3 c^2 \ldots\right) \qquad (1)$$

where π is the osmotic pressure, R the gas constant, T the temperature, c the insulin concentration (in mg/ml) and $\overline{M}_n$ the mean number average molecular weight . Γ represents the virial coefficient. π/c denotes the reduced osmotic pressure.

From fig. 1 it follows that the reduced osmotic pressure of cobalt (III) insulin hexamer is independent of the concentration within the accuracy of the measurements.

As the cobalt (III) insulin hexamer is an exchange inert complex and cannot dissociate [10], the lack of concentration dependence shows that the contribution from the virial coefficients in eq. 1 is negligible.

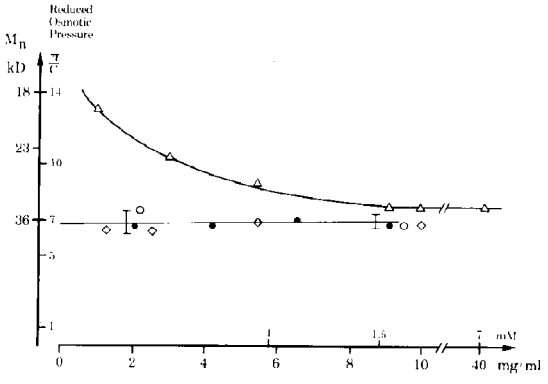

Fig. 1. Osmotic pressure of insulin as a function of concentration. Solvent: 0.05 M NaCl, pH 7.5, Temperature 25 °C. (△) Human insulin, Zn-free; (○) human insulin, 2 Zn per hexamer; (◇) insulin analogue B13-glutamine, Zn-free; (●) cobalt (III) human insulin hexamer.

The reason for the lack of virial coefficients must be that the terms in the second (and higher) virial coefficient (i.e., excluded volume, Donnan term, charge fluctuation term) give rise to no net contribution under the actual experimental conditions. A similar situation has been reported in the case of human serum albumin [12]. On ignoring the virial terms, eq. 1 reduces to:

$$\frac{\pi}{c} = \frac{RT}{M_n} \qquad (2)$$

If the effect of differences in charge of the various insulin species shown in fig. 1 is considered to be small, then it is possible to use eq. 2 to calculate the average molecular weight at a given concentration and, in principle, to evaluate the association constants. From fig. 1 the following results are obtained:

2-Zn and Co(III) human insulin hexamer and Zn-free insulin analogue B13-glutamine are hexameric throughout the whole concentration range. Zn-free human insulin associates to near hexamer (mol. wt. ≈ 33 000) at 1.5 mM, and up to at least 7 mM the molecular weight observed (35 000) represents the maximum association.

Fig. 2 depicts the pH dependence of Zn-free insulin. At pH 7.0, maximum molecular weight of 35 000 (hexamer) is observed at a concentration as high as 1 mM human insulin. A molecular weight of 40 000, slightly above the hexamer value is found at pH 6.8. The pH limit of precipitation in 50 mM NaCl was found to be close to pH 6.7 (examined by visual inspection).

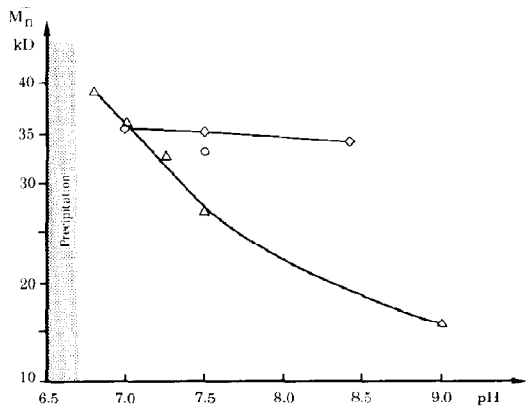

Fig. 2. pH dependence of insulin association. Solvent: 0.05 M NaCl, Temperature 25° C. (△) 1 mM human insulin, Zn-free; (○) 1.5–7 mM human insulin, Zn-free; (◇) 1 mM insulin analogue B13-glutamine, Zn-free.

4. Discussion

The general use of eq. 2 is based on the assumption that the influence of charge differences can be neglected.

The charge of Zn-free human insulin at pH 7.5 is approx. −24 per hexamer [13]. According to this value, 2-Zn human insulin hexamer has a charge of −20, while that of 2-cobalt (III) insulin hexamer and zinc-free insulin analogue B13-glutamine hexamer is −18.

From fig. 1, it can be seen that no measurable differences exist between the slopes of the plots for Co(III) insulin hexamer, 2-Zn human insulin and insulin analogue B13-glutamine hexamers. This result indicates that the neglecting of the virial terms in eq. 1 in the case of Zn-free human insulin also provides a good approximation of the association. Furthermore, the osmotic pressure results in values for the molecular weights at equilibrium without any approximations.

The finding that Zn-free insulin analogue B13-glutamine is hexameric in the millimolar concentration range demonstrates that Zn^{2+} is not necessary to form a hexamer, and that the hexamer is a natural association species. The difference as compared to human insulin is that B13-glutamate is replaced by B13-glutamine, i.e., there is a charged carboxylate in human insulin at physiological pH. In the hexamer these glutamates are close to each other [7], and result in the destabilization of the hexamer as demonstrated in fig. 1. Human insulin is predominantly hexameric at concentrations above 1 mM, in contrast to analogue B13-glutamine which is hexameric down to at least 0.2 mM. Since human insulin even at 7 mM does not exceed the hexameric aggregation state, there is no evidence of higher aggregation states than the hexamer at pH 7.5.

Decreasing pH increases the degree of association (fig. 2), however, only at pH 6.8, which is close to the pH of precipitation, is an association

state slightly higher than the hexamer observed. The reason for this association may be due to initial precipitation which is not visible.

Ultracentrifugation experiments resulted in a value for the molecular weight at pH 7.0 of 55 000 [1–5]. A possible explanation for the discrepancy vs the value of 36 000 found in the present work may be in relation to an initial stage of precipitation at pH 7.0, which may be enhanced by the high pressure in the ultracentrifuge. A small amount of high molecular weight aggregates that are still soluble before the visual detection of precipitation will have a greater influence on the weight average molecular weight measured by ultracentrifugation than on the number average molecular weight determined by the osmotic pressure.

The linear polymerization will have as a consequence that, on changing the pH of human insulin from 7.5 to 7.0 or replacement of B13-glutamate by glutamine, the mechanism of association should change from hexameric to linear association, which is unlikely to happen. Ultracentrifugation experiments in the pH range 7–8 may help to clarify these questions. The data on the osmotic pressure provide no evidence in favour of an association mechanism other than that of dimer to hexamer.

It is therefore concluded that measurement of the osmotic pressure is a suitable method for investigation of the association behaviour of insulin at millimolar concentrations in the physiological pH range, and that Zn-free insulin at physiological pH associates to the hexamer, the choice of method being very important when studying the association behaviour of proteins.

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