



Microcalorimetric studies of insulin and Zn(II)-insulin over a wide range of pH and protein concentration

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It is shown that the denaturation process of a medicinal preparation of insulin in dilute solution covers the temperature range 50–96 °C. The excess heat capacity (ΔC_p) increases linearly with temperature and is equal to 0.29 J g⁻¹ K⁻¹ at 62 °C. A significant peak with $T_d = 81.5$ °C, $\Delta H_d = 43.4$ J/g and $\Delta T_d = 13.5$ °C is observed in the temperature range 62–96 °C. Calculation of the ratio $\Delta H_d^{\text{cal}}/\Delta H_d^{\text{eff}}$ of calorimetric to effective (van't Hoff) enthalpies gives the value 0.83±0.1 which is fairly close to 1.0, indicating that the denaturation process of insulin proceeds as usually observed for compact globular proteins. The melting process of a medicinal preparation of Zn(II)-insulin, which is not an optically transparent solution, has three stages with $T_d^1 = 77.0$, $T_d^2 = 82.5$ and $T_d^3 = 91.5$ °C; all these stages correspond to various aggregation states of the protein. Hence it is inferred that Zn(II)-insulin exists in three different energetic states depending on concentration: in the concentration range 0.1–0.5%, ΔH_d decreases from 25.3 to 16.0 J/g; in the concentration range 1.5–40% is characterized by a constant value of ΔH_d equal to 16.0 J/g; in the concentration range 40–80%, ΔH_d drops monotonously from 16.0 to 5.2 J/g. It is evinced that this decrease of ΔH_d is connected with disruption of the protein hydration shell.

Keywords: calorimeter, denaturation, enthalpy, insulin

1. INTRODUCTION

A great number of calorimetric measurements have been carried out for obtaining the thermodynamic parameters of protein denaturation process [11]. It was established that the number of hydrogen bonds in small and large globular proteins correlated with molecular weight is practically identical but the specific number of contacts and the energy of interaction between nonpolar groups decreases as the temperature increases and is described by a linear dependence of the enthalpy of protein denaturation on temperature $\Delta H_d = f(T_d)$. It was furthermore found that peptides built from 30 to 60 amino acid residues only partially conform to this law because their small size prevents them from forming a perfect hydrophobic nucleus, and, in consequence, a unique tertiary structure [1]. Because of peculiarities of their structural organization, it is difficult to study these peptides even with highly sensitive microcalorimeters,

due to the low value of the melting enthalpy and the extremely wide melting range. It should also be noted that the measurements may be carried out only with dilute solutions because of certain peculiarities of the construction of modern highly sensitive microcalorimeters [10].

Many proteins, including the insulin found in the beta granules of the pancreas [9], are in the form of crystals having a hexagonal structure, whose compactness, and whose transformation from (in this case) proinsulin to insulin by the spallation of the C peptide, are dependent on the pH of the surrounding medium.

In this work we present results on the thermal denaturation of a medicinal preparation of insulin (Actrapid) and Zn-insulin over a wide range of solution pH and biopolymer concentration.

2. MATERIALS AND METHODS

Measurements were carried out using a laboratory-built differential scanning microcalorimeter with a sensitivity of 10⁻⁷ W [7] designed for the study of complex biological systems. The volume of the measuring cell was 0.29 cm³, the heating rate 1–100 °C/hour, and the temperature range of the measurements was 5–140 °C. The exactness of the temperature measurements was at least 0.05 K.

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Insulin (Actrapid HM Penfill, Novo Industri, Denmark) was used in all experiments. The pH of the solutions were adjusted where necessary by adding 0.1 M HCl or NaOH.

3. RESULTS

Fig. 1 shows a heat absorption curve of dilute insulin solution at pH 7.0. The melting process covers a temperature range of 50–96 °C. This range may be divided into two parts. The first is from 50–62 °C, in which the excess heat capacity (ΔC_p) increases monotonically and has a value of 0.29 J g⁻¹ K⁻¹ at 62 °C. The second part is observed in the temperature range 62–96 °C, with a maximum at 81.5 °C, with the width at half maximum of the peak (ΔT_d) equal to 13.5 °C, and the melting heat $\Delta H_d = 43.4$ J/g.

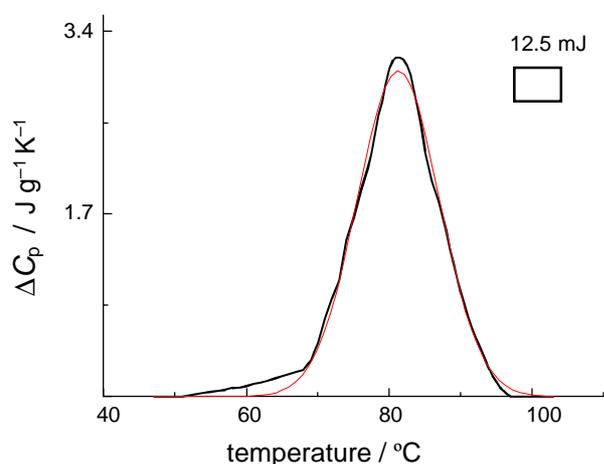


Figure 1. Heat absorption curve of dilute insulin solution (pH 7.3) normalized to 1 g of protein dry weight. Heating rate: 12 °C/h. The faint line is the deconvolution curve.

Changing the pH by one unit towards either acid or alkaline values does not influence the heat absorption curve profile, the integral heat, nor the melting temperature. But further movement of pH causes significant changes. At pH 4.0, the heat absorption process covers the temperature range 40–96 °C and is characterized by maxima at about 70 and 82.5 °C and a shoulder around 57 °C. The temperature (T_d) at which half of the insulin molecule is in an ordered helical state and the other half in the melted coil state is 70 °C. A change of the heat absorption profile and a decrease of the melting temperature were observed at alkaline pH.

Fig. 2 presents heat absorption curves of Zn(II)-insulin at various pH values. It is clearly seen that the denaturation process of Zn(II)-insulin has many stages.

As the aqueous solutions of Zn(II)-insulin are turbid mixtures (suspensions), the observed stages

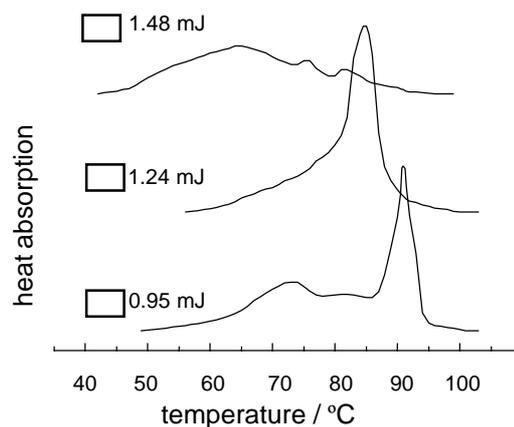


Figure 2. Heat absorption curves of dilute insulin solution, normalized to 1 g of protein dry weight, at different pH values: from bottom to top, 7.3; 4.0; and 3.1.

of heat absorption belong not to the melting of individual molecules but to the denaturation of macromolecules present as various aggregates.

As it seen is from Fig. 2, the most ordered aggregate is formed at pH 7.3, the specific part of which is equal to 55 % of the total quantity of substance. Denaturation of this fraction is characterized by the parameters $T_d = 91.0$ °C, $\Delta T_d = 3.0$ °C and $\Delta H_d = 14.1$ J/g. The change of pH by one unit in either the acid or the alkaline direction caused a sharp change in the profile of the denaturation curve without changing the integral heat of denaturation. Further changes of pH caused a smoothing of the curves and a decrease of the integral heat of denaturation.

Increasing the biopolymer concentration or removing water from the samples (which amounts to the same) influences the denaturation parameters of Zn(II)-insulin, as shown in Fig 3. As can be seen from Fig. 4, there is a sharp decrease of ΔH_d (from 25.2 to 16.0) over the concentration range 0–0.4 %; constancy of ΔH_d over the concentration range 0.4–40 %, and a monotonous decrease of ΔH_d over the concentration range 40–80 %, down to 5.2 J/g at 80 %.

4. DISCUSSION

A linear dependence of denaturation specific enthalpy on temperature is observed for a set of small compact globular proteins (Fig. 5) [11], extrapolation of which to 110 °C leads to the same value of the enthalpy (54.2 J/g). This value is the conformational enthalpy of protein denaturation, which includes the rupture of weak intramolecular bonds including S–S bonds. It is known that if a biopolymer denaturation process really is a two-stage transition, the effective (van't-Hoff) enthalpy ΔH^{eff} must be equal to ΔH_d^{cal} i.e. $\Delta H_d^{\text{cal}}/\Delta H^{\text{eff}} = 1.0$. The

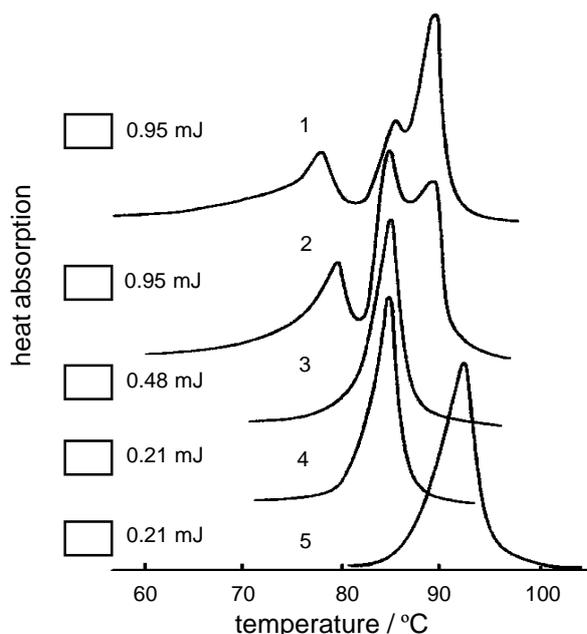


Figure 3. Heat absorption curves of Zn-insulin at pH 7.3, normalized to 1 g of protein dry weight, at different protein concentrations: 1, 0.2 %; 2, 0.4 %; 3, 1.0 %; 4, 38.5 %; 5, 80.0 %.

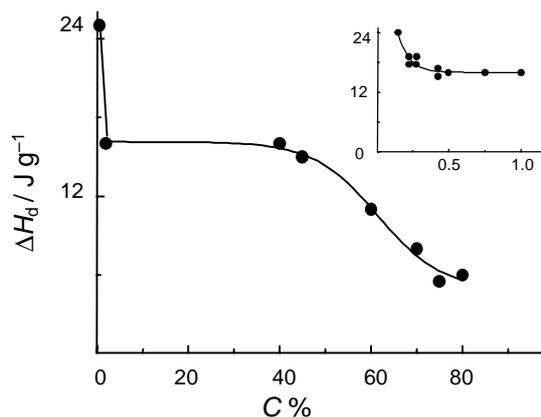


Figure 4. Denaturation enthalpy of Zn-insulin vs protein concentration C at pH 7.3. Inset: expansion of the low concentration region.

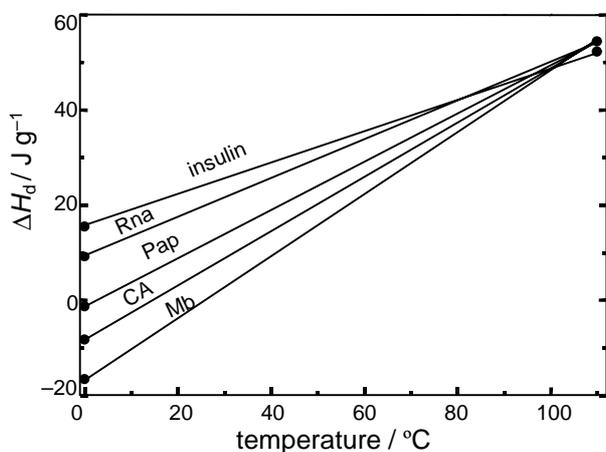


Figure 5. Dependence of denaturation enthalpy (ΔH_d) on T_d for Mb (Myoglobin), CA (carbonic anhydrase), Pap (papaain), Rna (RNAase [11]) and insulin (our data).

effective enthalpy is connected with the calorimetric one by the following equation (ref. [11], p. 183):

$$\Delta H^{\text{eff}} = 4 R (273 + T_d)^2 \Delta C_d^{\text{max}} / \Delta H_d$$

ΔH_d is the enthalpy of protein denaturation, T_d the temperature corresponding to the heat absorption peak maximum, and ΔC_d^{max} is the excess heat capacity corresponding to the maximum of the heat absorption peak. Putting the experimentally obtained denaturation parameters at pH 7.3 into equation (1): $\Delta C_d^{\text{max}} = 3.10 \text{ J g}^{-1} \text{ K}^{-1}$, $T_d = 354.5 \text{ }^\circ\text{C}$, $\Delta H_d = 43.5 \pm 4.5 \text{ J/mol}$, we obtain the value 71.5 kJ/mol for ΔH^{eff} , and hence the ratio $\Delta H_d^{\text{cal}}/\Delta H^{\text{eff}}$ is equal to 0.83 ± 0.1 .

A small but significant deviation of that ratio from 1.0 ± 0.05 points to the partial aggregation of insulin. A similar effect (decrease of the calorimetric enthalpy of denaturation) was observed for RNAase (barnase), because of weak association of barnase molecules during the heating process [6]; extrapolation of $\Delta H_d = f(T_d)$ to $110 \text{ }^\circ\text{C}$ gives a value of ΔH_d^{conf} equal to 51.8 J/g , which is 5 % less than ΔH_d^{conf} obtained for many other compact globular proteins, whose denaturation occurs according to the ‘‘all-or-none’’ principle.

A different picture is observed in the denaturation of Zn(II)-insulin, which even at a concentration of 0.2 % is a turbid mixture. Therefore, the observed heat absorption stages (Fig. 2) belong not to the melting of individual, isolated insulin molecules, but to the melting of various aggregate forms. Our data show that Zn(II)-insulin in physiological medium and at a low concentration (pH 7.3, 0.2 %) forms three aggregate forms in various degrees of organization.

The rôle of the Zn(II) ion in the formation of a unique tertiary structure of proteins [13] and peptides [8], enabling them to carry out their functions *in vivo*, is well documented. It has also been established that the process of thermal denaturation of lactate dehydrogenase M_4 (LDH) [2] and carbonic anhydrase B [12] in the presence of low concentrations of Zn(II) ions is characterized by lower values of ΔH_d^{cal} due to weak aggregation of the protein during the melting process. These data have something in common with our results shown in Figs 3 and 4, in particular with the decrease of ΔH_d from 25.2 to 16.0 J/g over a narrow range of protein concentration, and may be understood by reference to [3–5], according to which the ΔH_d decrease of ordered protein aggregates, fibres and protein crystals is connected with crosslinking of intra- and intermolecular hydrogen bonds but not with molecules of water as occurs in the case of dilute

solutions. The invariability of ΔH_d , T_d and ΔT_d (Fig. 3, curves 3 and 4) over the protein concentration range 0.5–40 % (Fig.4) obviously shows that the aggregate formed does not change its structure in this range. As for the monotonous decrease of ΔH_d (16.0–5.1 J/g) in the protein concentration range 40–80 %, it is evidence for the gradual destruction of the hydration shell of the protein, which normally helps to stabilize tertiary protein structure.

The high value of hydration obtained for Zn-insulin (0.6 g H₂O/g protein) indicates a looser tertiary structure, which allows solvent to penetrate the protein globule [14].

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