ENERGETICS OF CYCLODEXTRIN-INDUCED DISSOCIATION OF INSULIN

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ABSTRACT

The energetics of dissociation of bovine insulin oligomers in aqueous solution under various conditions have been investigated by dilution microcalorimetry. Addition of cyclodextrins increases dissociation of insulin oligomers in solution in a manner consistent with interaction of these cyclic polysaccharides with protein side chains. For example, assuming monomer-dimer equilibrium, in the absence of cyclodextrins dilution data (25 °C, pH 2.5) are consistent with a dimer dissociation constant (K_{diss}) of about 12 μM and a dimer dissociation enthalpy (ΔH_{diss}) of +41 kJ mol^{-1}. Addition of methyl-β-cyclodextrin (up to 200 mM) makes dissociation significantly more endothermic (ΔH_{diss} = 79 kJ mol^{-1}) and reduces the apparent dimer dissociation constant by more than two orders of magnitude ($K_{diss} \approx 1.7$ mM). Qualitatively similar results are observed with α-cyclodextrin and other β-cyclodextrin derivatives.

1. INTRODUCTION

Insulins are known to occur in a variety of aggregation (oligomer) states in solution depending on concentration, pH, temperature, Zn^{2+} concentration and other ionic conditions [1], and their aggregation states can potentially affect their use in therapeutic situations. Based on previous observations on interaction of cyclodextrins with globular proteins [2-4] we predicted that complexation of these cyclic polysaccharides with surface protein residues might significantly affect the state of aggregation of protein in solution. The non-polar cavities of toroidal cyclodextrin molecules have a particular affinity for small non-polar groups and can both enhance the solubility and improve stability of such molecules in water. Cyclodextrins are finding increasing use as solubilizing agents and stabilizing excipients for protein drugs, including insulins [5]. We show here by calorimetric measurement of heats of dilution that the dissociation of bovine insulin in solution is significantly enhanced in the presence of various cyclodextrins. The energetics of this process are consistent with association of

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cyclodextrin molecules with insulin surface residues. Such a complexation might also bring about conformational changes in insulin which might contribute indirectly to the disaggregation process.

2. MATERIALS AND METHODS

Bovine insulin concentrations were verified by UV absorbance measurements on diluted aliquots assuming a molar extinction coefficient (ε_{280}) of 5734 M^{-1} [6]. Buffers used were 0.1M glycine/HCl pH 2.5 or 0.1M Na-phosphate pH 7.4, containing appropriate concentrations of cyclodextrins where required. Calorimetric dilution experiments were done using a Microcal OMEGA titration microcalorimeter following standard instrumental procedures at 25°C [7,8]. In a typical dilution experiment small aliquots (10-20µl) of concentrated insulin, dissolved in buffer or buffer/cyclodextrin mix, were injected into the calorimeter reaction vessel containing the identical buffer mixture. Integrated heat pulse data, after correction for mixing controls done separately under identical conditions, were analysed by non-linear regression in terms of a simple monomer-dimer equilibrium model to give the apparent equilibrium constant (K_{diss}) and enthalpy of dissociation (ΔH_{diss} per mole dimer).

3. RESULTS AND DISCUSSION

Dilution of a series of small aliquots of insulin into a larger volume of buffer in the microcalorimeter gives a sequence of endothermic heat pulses characteristic of molecular dissociation (Fig.1A). A typical heat of dilution curve which can be fitted in terms of a monomer-dimer equilibrium model yielding K_{diss} and ΔH_{diss} is shown in Fig.1B. Addition of cyclodextrins to the buffer mixture gives rise to two significant effects (Fig.1C): (i) insulin dilution heats become more endothermic, and (ii) the dilution curves become more attenuated, indicating greater dimer dissociation in the presence of cyclodextrins.

Previous studies have shown that, at low pH, insulin is predominantly dimeric in solution at high concentrations [1]. In the absence of cyclodextrins at pH 2.5 the insulin dimer dissociation constant (K_{diss}), obtained from non-linear regression analysis of dilution data, is around 12µM and in agreement with previous determinations by other techniques [1]. At pH 7.4 the oligomeric state of insulin is less clear, and hexamers or higher oligomers almost certainly exist under the conditions used here. Nevertheless, the dilution data fit reasonably to a dimer model.

Non-linear regression analysis of experimental data for the variation of apparent dimer dissociation constant with cyclodextrin concentration (Fig.2) indicates that two sequential binding sites are adequate for describing the data satisfactorily over the accessible concentration range.

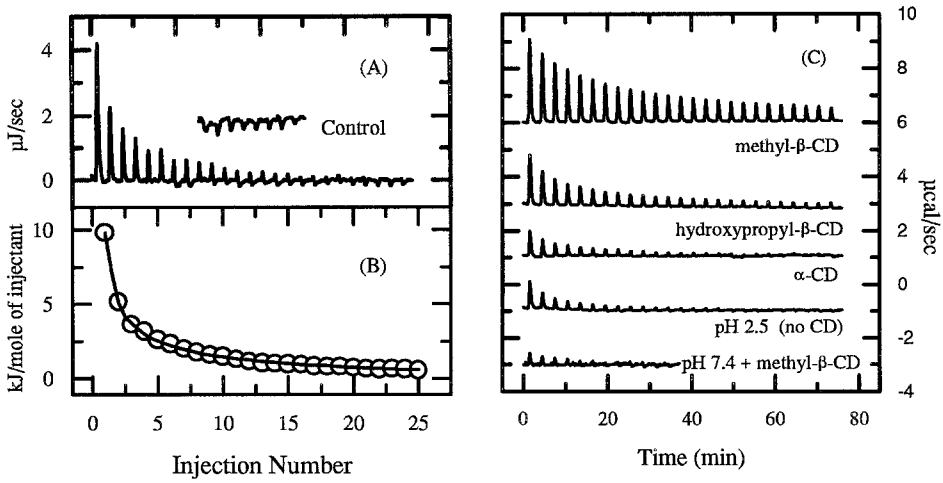

Fig. 1 Calorimetric data for the endothermic dissociation of insulin dimers at pH 2.5: (A) Raw data for injection of insulin, 1.53mM, 25 × 10 μl injections, into buffer at 25 °C, with control data; (B) Integrated injection heats, corrected for control heats and fit (solid line) to a dimer dissociation model with K_{diss} = 12μM and ΔH_{diss} = 41 kJ mol^{-1}; (C) Examples of raw calorimetric dilution data showing the effects of different cyclodextrins on insulin dissociation, all at pH 2.5 except where indicated. For comparison, cyclodextrin concentrations (when present) are all approximately 100 mM in this case.

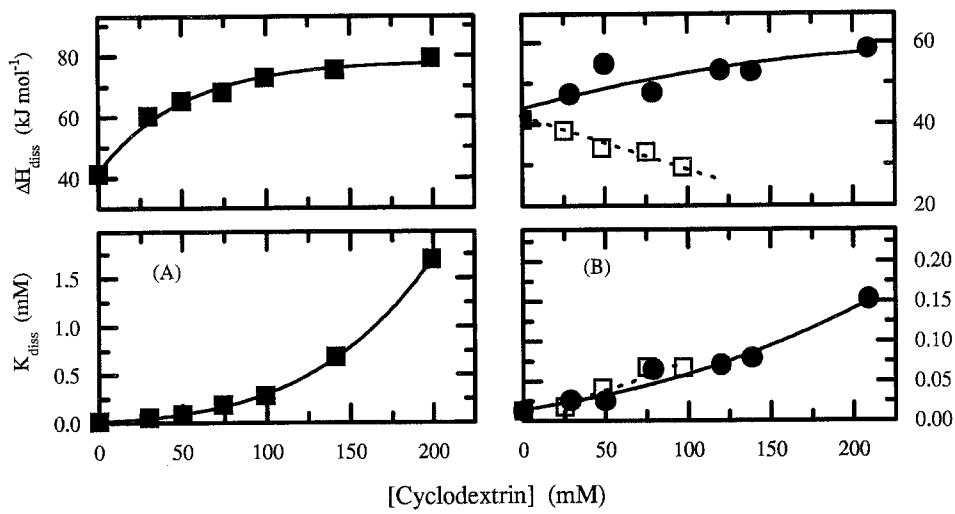

Fig. 2 Change in apparent dissociation constants (K_{diss}, lower panels) and enthalpies of dissociation of insulin (ΔH_{diss}, upper panels) at pH 2.5 with increasing cyclodextrin concentrations. In the lower panels the curves show the theoretical fits to a simple sequential binding model. (A) Methyl-β-cyclodextrin (filled squares). (B) Hydroxypropyl-β-cyclodextrin (filled circles) and α-cyclodextrin (open squares).

The numerical values obtained for the site-binding constants (K_1, K_2) are consistent with the relatively weak affinities expected on the basis of previous observations of interaction between cyclodextrins in solution and aromatic amino acid side chains and similar groups [2,9,10]. For methyl-β-cyclodextrin, which shows the biggest effect, K_1 and K_2 are estimated to be about 20 and 6.5 M^{-1}, respectively. For the other cyclodextrins $K_1 \approx 10\text{-}15\ M^{-1}$, with $K_2 < 5\ M^{-1}$.

4. CONCLUSION

In the absence of cyclodextrins the dissociation of insulin oligomers is endothermic. Addition of α-cyclodextrin, in addition to encouraging oligomer dissociation, also makes this dissociation less endothermic in a manner consistent with the exothermic binding of α-cyclodextrins to exposed groups on insulin monomers after dissociation. In contrast, although methyl- and hydroxypropyl-β-cyclodextrins similarly induce oligomer dissociation, this dissociation is observed to be more endothermic. This suggests that the binding of these modified β-cyclodextrins to exposed insulin residues, although thermodynamically favourable, is endothermic and consequently entropy-driven.

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