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A sedimentation equilibrium study of platypus insulin: the HB10D mutant does not associate beyond dimer

Amanda Nourse, Peter D. Jeffrey *

Protein Chemistry Group, Division of Biochemistry and Molecular Biology, The John Curtin School of Medical Research, Australian National University, GPO Box 334, Canberra, ACT, 2601, Australia

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Abstract

An extensive study of the self-association patterns of zinc-free synthetic native and mutant (HB10D) platypus insulin in solution (pH = 7.0; $I = 0.1$ M; 25°C) has been undertaken using the method of sedimentation equilibrium. The data was fitted to a mathematical equation describing the indefinite duoisodesmic (IDI) model of self-association [A.E. Mark, P.D. Jeffrey, Biol. Chem. Hoppe-Slayer, 371 (1990) 1165]. From this the relevant association constants, K_A and K_B, describing the polymerising system were calculated. This information allows the calculation of the complex distribution of odd and even numbered polymeric species within the insulin system in solution. In the studies on the self-association of the synthetic native and mutant platypus insulins, each was compared with bovine insulin as well as with each other. It is concluded that there is some reduction in the extent of the self-association of native platypus insulin compared to bovine insulin. A reduction, in specifically the dimer–dimer interaction, is indicated by the higher K_A and lower K_B values. HB10D platypus insulin shows a dramatic reduction in self-association compared to native platypus and to bovine insulin. Analysis of the self-association pattern yielding a K_B value of effectively zero suggests that the substitution of an aspartic acid residue for a histidine at B10 virtually abolishes its dimer–dimer interaction. Platypus insulin has essentially the same biological activity as that of porcine (submitted for publication) but a somewhat lower self-association, while the introduction of one amino acid in a critical region increases the activity twofold while abolishing self-association beyond dimer. © 1998 Elsevier Science B.V.

Keywords: Platypus insulin; HB10D platypus insulin; Insulin association pattern; Indefinite self-association; Sedimentation equilibrium

1. Introduction

Insulin self-associates reversibly in a zinc-free solution characteristically forming an array of polymeric species in equilibrium [1,2]. In the presence of zinc, hexamers are the most prominent polymeric species. It has been suggested that this stable zinc hexamer exists together with background polymerisation of zinc-free insulin [3]. The zinc hexamer is the form in which insulin molecules are stored as inactive crystals in the pancreas [4]. In solution, hexamers are evident at quite low concentrations even in the absence of zinc [1]. Many of the problems associated with the exogenous administration of insulin originate as a result of self-association at the

* Corresponding author. Tel.: +61-06-2493652; fax: +61-06-2490415; e-mail: peter.jeffrey@anu.edu.au

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high application concentrations that are used [5–8] and this is an ongoing problem in insulin therapy [9]. These aggregates engage the active monomers in interactions to form biologically inactive polymers [10].

At present, several insulin analogues that are thought to be predominantly monomeric have and are being investigated for potential therapeutic use [8]. The strategies that were, to date, employed to achieve this monomeric state were to substitute or modify residues in the interfaces between molecules in dimers and higher polymers, the so-called monomer–monomer interface and the dimer–dimer interface, so as to reduce self-association without adversely affecting the activity of the insulin [11–13]. With some of the analogues tested, it is claimed that they display clinically similar pharmacokinetics on injection (subcutaneously) at meal times to the endogenous insulin secretion seen in response to a meal [14,15].

Some of the methods used to assess the association states of these insulin analogues are relatively insensitive. An illustration of this is the use of osmometry for assessing polymeric status by the NOVO group [11] where it is claimed that some of these analogues were mostly monomeric in solution. A close examination of this work led to the conclusion that although good progress had been made in increasing the amount of monomeric species at concentrations used for insulin formulations, the monomer was not the major molecular species at the chosen concentration [16,10]. This emphasises the need for a more precise method to study critical quantitative changes to self-association as well as to potency. To date, none of these 'monomeric' insulins have been implemented in insulin therapy [8].

Since reducing or abolishing the self-association of therapeutic insulin is pivotal to improving insulin therapy, it is important to be able to analyse critically the self-association pattern of insulin analogues for potential use in solution. It is thus crucial that a reliable and reproducible method for measuring self-association behaviour be used to monitor molecular changes designed to modify this behaviour. It is argued here that sedimentation analysis in the analytical ultracentrifuge is still the best technique for characterising protein interactions in solution [10]. The self-association pattern of insulin analogues may be quantitatively compared using a model of self-association that best describes the system and by calculating association constants that describe the relative strengths of interaction at the two association interfaces. In this study, sedimentation analysis with the analytical ultracentrifuge has been used for evaluating chemically synthesised insulins in detail. Association constants, characteristic of the self-association pattern of a specific insulin, were derived using this analysis allowing quantitative assessment of the effects of sequence changes.

1.1. Exploring another avenue for identifying an insulin with desirable properties

The purpose of the present undertaking was to explore another avenue for identifying an insulin with desirable properties as a template, namely to exploit natural variation as exhibited in an exotic species like the platypus. Modifications of self-association behaviour without loss of activity may succeed in one of the more exotic insulins because its different composition and properties offer a different baseline for modification from the common mammalian insulins.

In this study, the platypus insulin sequence was used to explore and identify desirable properties for use in insulin therapy. The platypus (*Ornithorhynchus anatinus*), like mammals, has mammary glands but lays eggs [17,18]. Based on its sequence [19], platypus insulin was chemically synthesised in a pure form that displayed full activity (submitted for publication). The total chemical synthesis of platypus insulin yielded sufficient material for preliminary investigations into its self-association pattern. These investigations pointed to some reduction in the self-association at the dimer–dimer interface compared to bovine insulin. Furthermore, predicted differences in the spatial arrangement of residues in the dimer–dimer interface of the three-dimensional molecular model [19] also suggested that the self-associating properties in this region may be affected, seemingly lowered. Accordingly, a platypus insulin mutant (B10H is replaced by an Asp) was synthesised with a substitution in the hexamer forming region (dimer–dimer interface). The effect of this mutation on the self-association pattern of platypus insulin is analysed and investigated in detail in this paper. The

study of this mutant was motivated by the reduced self-association properties and increased biological activity (in vitro) found for the HB10D mutant of human insulin [13,20].

Sedimentation analysis of the self-association patterns of these chemically synthesised insulins was undertaken in a zinc-free solution, at room temperature and neutral pH. The sedimentation equilibrium method was chosen because of the accurate weight average data over a concentration range that can be obtained. The omega method of analysis was used to obtain the thermodynamic parameter a_1 (the activity of the insulin monomer in equilibrium with a series of polymeric species) as a function of total insulin concentration. As shown later, this approach allows quantitative analysis of the sedimentation equilibrium data in terms of a model self-association pattern and the corresponding equilibrium constants.

2. Theory

2.1. The omega analysis

Omega analysis permits the evaluation of the thermodynamic parameter a_1, the activity of the monomer, in equilibrium with a series of polymeric species, as a function of total weight concentration $\bar{c}$ [21]. The omega function (Ω) provides a means of fitting reaction models to sedimentation equilibrium data obtained with polymerising systems. It is an alternative to fitting the concentration vs. radial distance data written in terms of specific models for the self-association process and was used in the present work because of our success with it in previous analyses of sedimentation equilibrium data obtained with insulin.

The experimentally obtained plot of $\bar{c}$ vs. r may be analysed to yield the activity of the monomer a_1 as a function of total weight concentration $\bar{c}$ [21]. This is achieved by defining an experimentally determinable dimensionless function $\Omega(r)$ as:

$$\Omega(r) = \frac{\bar{c}(r)\exp\left[\phi_1 M_1\left(r_F^2 - r^2\right)\right]}{\bar{c}(r_F)} \qquad \phi_1 = \frac{\left(1-\bar{\nu}_1\rho\right)\omega^2}{2RT} \tag{1a}$$

$\bar{\nu}_1$ is the partial specific volume of the monomer, ρ the solution density, ω^2 the angular velocity, R, the universal gas constant and T the absolute temperature of the sedimentation equilibrium experiment. M_1 is the monomer molecular weight, (r) and (r_F) the total protein concentrations at radial positions r and r_F between or at r_m and r_b, the meniscus and base of the cell, respectively.

Provided M_1 and $\bar{\nu}_1$ are known and any reference radial distance, r_F, is chosen to lie between or at r_m and r_b, a plot construct of $\Omega(r)$ vs. $\bar{c}(r)$ from experimental results can be obtained (in the case of the Beckman Optima XL-A from absorption optical measurements) across the entire distribution. Raw data sets are measurements of $c(r)$ vs. radial distance r. By definition:

$$\Omega(r) = 1 \text{ at } \bar{c}(r_F) \tag{1b}$$

and may be rewritten as:

$$\Omega(r) = \frac{a_1(r_F)\bar{c}(r)}{a_1(r)\bar{c}(r_F)} \tag{1c}$$

Eqs. (1a), (1b) and (1c) make it possible to evaluate $a_1(r)$ as a function of $\bar{c}(r)$, a useful relationship with homogeneous systems as it allows the activity coefficient to be determined experimentally. The value of $\bar{c}(r_F)$ used to compute $\Omega(r)$ may be selected as any value in the observed total concentration range.

As $\bar{c}(r) \to 0$, $a_1(r) \to 0$ since the activity coefficient tends to unity and in addition, $\bar{c}(r) \to a_1(r)$ since dilution favours dissociation. A plot of $\Omega(r)$ vs. $\bar{c}(r)$ can be extrapolated to infinite dilution to yield a value for Ω^0.

$$\lim_{\bar{c}(r)\to 0} \Omega(r) = \Omega^0 = \frac{a_1(r_F)}{\bar{c}(r_F)} \tag{2}$$

Since the selected value of $\bar{c}(r_F)$ is known, the monomer activity, $a_1(r_F)$ can be calculated. The value of $a_1(r_F)$ is then used to determine the monomer activity, $a_1(r)$, at any radial position and hence, at any measured total concentration $\bar{c}(r)$. Eqs. (1a), (1b), (1c) and (2) may be rewritten in terms of $a_1(r)$ as follows:

$$a_1(r) = a_1(r_F \exp\left[\phi_1 M_1\left(r_F^2 - r^2\right)\right] \tag{3}$$

allowing a_1 to be calculated as a function of r.

An experimental plot of $a_1(r)$ vs. $\bar{c}(r)$ can now be constructed and fitted with various models for the self-association written in the same terms. In a 1990 paper, Mark and Jeffrey [16] surveyed various models for insulin self-association and experimental data available for the process under all conditions investigated. They concluded that the indefinite duoisodesmic (IDI) model was the only one that was satisfactory when non-ideality corrections were included in the analysis. Thus, the IDI model is the favoured model, both on the grounds that it is most consistent with the available information from structural studies and that it is the best fit to the experimental data. Accordingly, it is the one applied to the experimental data reported in this paper for platypus insulin and the HB10D mutant platypus insulin.

The model, called indefinite duoisodesmic (IDI), describes the indefinite self-association of a bivalent insulin monomer along the monomer–monomer and dimer–dimer interfaces governed by two binding constants, K_A and K_B, respectively. The two independent nonidentical self-association sites are designated α (monomer–monomer interface) and β (dimer–dimer interface), both capable of self-interaction. The two initial pathways of dimerization are followed by the indefinite addition of monomer under the control of the same two equilibrium constants.

Two types of dimers are formed, one involved in an α–α interaction, leaving the two β sites exposed and governed by an association constant K_A; the other involving β–β interaction, leaving two α sites exposed and governed by an association constant K_B. Linear chain growth proceeds by successive addition of monomer so that all polymers, both odd- and even-numbered, coexist in equilibrium. Each of these polymers possesses alternating α–α and β–β bonds with even numbered polymers having either two α-sites or two β-sites exposed and odd numbered polymers having an α-site at one end and β-site at the other.

In the original presentation of this model, the conclusion made by the authors [1] was that the α–α interactions may be identified with those between monomers about the OP crystallographic axis [4,22] and that the β–β interactions most certainly exist in solution. Thus, it is possible to describe the solution behaviour of zinc-free insulin in terms of a single association pattern at neutral pH and 25°C. In general, the solution will contain an equilibrium mixture of all polymeric forms of insulin of which the composition depends on the total concentration and the values of K_A and K_B for a specific environment.

The mathematical equation developed by Nichol et al. [23] describing this model taking both K_A and K_B into consideration and distinguishing between the nonidentical self-association domains is:

$$\bar{c} = M_1 m_1 \left[\frac{(1 + 2K_A m_1)(1 + 2K_B m_1)}{\left(1 - 4K_A K_B m_1^2\right)^2} \right] \quad (5)$$

where M_1 is the molecular weight of the monomer, m_1 its concentration on the molar scale and $\bar{c}$ is the total protein concentration in g l^{-1}. Thus, the above mathematical equation describes the composition of a solution consisting of an infinite array of odd- and even-numbered polymeric species and can be used as the basis of a fitting process once the m_1 vs. $\bar{c}$ data is available from the application of the Ω analysis. The weight-fraction of each species can be calculated as a function of total insulin concentration.

Mark et al. [1] developed an expression describing the dependence of 'reduced' weight-average molecular weight ($\overline{M}_w/M_1$) on total concentration $\bar{c}$:

$$\overline{M}_w/M_1 = P/Q, \quad (6a)$$

where:

$$P = 16K_A^2 K_B^2 m_1^4 + 16K_A K_B (K_A + K_B) m_1^3 + 24K_A K_B m_1^2 + 4(K_A + K_B) m_1 + 1, \quad (6b)$$

$$Q = \left(1 - 4K_A K_B m_1^2\right)(1 + 2K_A m_1)(1 + 2K_B m_1) \quad (6c)$$

Eqs. (5) and (6a) describe a smooth monotonically increasing dependence of $\overline{M}_w/M_1$ on $\bar{c}$ and can be used to calculate this dependence without resorting to a differentiation procedure.

2.2. Weight-fractions of species

Mark et al. [1] also formulated an expression in terms of the weight-fraction of species as a function of total concentration for the IDI model. The weight-fraction of the insulin species present in solution under the relevant conditions as a function of total insulin concentration can be calculated provided K_A and K_B are known.

The weight-fraction, ϕ_i, of each species, i being given by:

$$\phi_i = \frac{c_i}{\bar{c}} = \frac{iX_i m_1^{i-1}\left(1 - 4K_A K_B m_1^2\right)^2}{(1 + 2K_A m_1)(1 + 2K_B m_1)} \quad (7a)$$

where:

$$X_i = (4K_A K_B)^{(i-1)/2} \quad i = \text{odd}, \quad (7b)$$

$$X_i = (K_A + K_B)(4K_A K_B)^{(i-2)/2} \quad i = \text{even}. \quad (7c)$$

The complex distribution of species within the insulin system in solution can thus be represented graphically. A distinction can be made between the weight-fraction of the two types of dimer α and β and tetramer α and β.

Thermodynamic non-ideality effects are assessed basically in terms of co-volume and charge–charge interaction effects. Non-ideality effects in the present system are not large due to the relatively small size of insulin, the small net charge borne by it and the low concentration range (0–4 mg ml^{-1}) that was examined [1]. Insulin is insoluble at higher concentrations in these solution conditions. Over the range of concentration for which the platypus data were available, the magnitude of non-ideality effects is insignificant in comparison with experimental error.

It is important to employ a model for the self-association process that define as precisely as possible the types and proportions of insulin species present in solutions of the protein under a given set of conditions in a way that reflects as closely as possible the real state of affairs. This information is important in the design of strategies to suppress insulin polymerisation completely while retaining full activity in the modified monomer. The IDI model has previously been shown to satisfy the basic requirement and, as will become evident, is also appropriate to the interaction between platypus insulin molecules.

3. Materials and methods

3.1. Materials

Glassware that had been in contact with the insulin samples was treated with 0.1 mM EDTA for 24 h to remove contaminating divalent cations followed by thorough rinsing in HPLC grade distilled water. Protein and peptide concentrations were determined spectrophotometrically using a Varian series 634 spectrophotometer. All dialysis was performed at 4°C using Spectrapor No. 3 membrane tubing (M_w cutoff 3500 Da). The tubing was thoroughly rinsed with HPLC grade distilled water. Solutions were dialysed against a minimum of 100 volumes of buffer for at least 24 h with the buffer being changed at least twice. Experimental solutions were allowed to equilibrate for at least 10 h after the final buffer change before ultracentrifugation. Three insulin preparations were used in these self-association studies. These were bovine insulin, totally chemically synthesised platypus insulin and totally chemically synthesised HB10D platypus insulin.

3.2. Sedimentation equilibrium studies

Bovine insulin (Sigma) (approximately 10 mg) was purified according to the method of Nourse et al. (submitted for publication) and lyophilised. This product was used in all the sedimentation equilibrium experiments. Only samples judged to be homogeneous by HPLC, mass analysis and high performance capillary electrophoresis were used in the experiments. The composition of the buffer employed in all the sedimentation equilibrium studies was as follows: 20 mM Tris, 18 mM HCl, 79 mM NaCl, 1 mM EDTA, pH 7.0, ionic strength, I, 0.1 M. The concentrations of the insulins in solution were determined spectrophotometrically at 276 nm employing an extinction coefficient of 10.5 for a 1% solution, 1 cm light path length [24]. These were substantiated when measured in a Beckman Optima XL-A using its optical system and a light path length of 1.2 cm during sedimentation equilibrium studies. Dilutions, when necessary, were performed with dialysate.

Sedimentation equilibrium experiments were carried out in a Beckman Optima XL-A Analytical Ultracentrifuge (Beckman Instruments, Fullerton, CA, USA) and final sedimentation equilibrium concentration distributions were obtained from scans at 280 nm using its absorbance optical system. Dialysed solution (100 μl) and dialysate (110 μl) were

placed in the sample and solvent sector, respectively, of a double sector carbon filled upon centrepiece. Experiments were conducted at different speeds, namely: 30 K rpm for 6 h; 42 K rpm and 50 K rpm for 12 h. The initial concentrations varied from 0.25 to 1.38 g l^{-1}. Baseline scans at 360 nm were subtracted to remove the effects of oil droplets on lenses and windows, as well as of optical imperfections in the windows and lenses.

3.3. Analysis of sedimentation equilibrium data

The computer program XLACALC/RALSTON/XLAMW was employed to calculate the weight-average molecular weight, $\overline{M}_w(r)$ as a function of total insulin concentration $\bar{c}(r)$ at the radius, r, after the baseline has been subtracted with the computer program XLACALC/RALSTON/XLABASE. RALSTONMW applies a 15 point sliding fit to the ln c vs. r^2 data and evaluates point average molecular weights as equal to $(\mathrm{d}\ln c/\mathrm{d}r^2)_r/\phi$. The solution density ρ has been taken as 1.00 g ml^{-1} and it has been assumed that $\bar{\nu}$, the partial specific volume of insulin, is the same for all insulin species namely the measured value, 0.725 ml g^{-1} [25,26]. Non-ideality effects were not taken into account in the calculations used in this work but $\overline{M}_w(r)$ was used rather than $\overline{M}_{w,app}(r)$ for convenience in nomenclature. Detailed analysis of sedimentation equilibrium data obtained with insulin solutions previously over a wide range of conditions showed that non-ideality effects are insignificant over the concentration range investigated here.

Omega values as a function of total concentration were calculated using Eq. (1a). A reference concentration of $\bar{c}(r) = 0.50$ g l^{-1} was chosen for all omega analyses. The value of the reference concentration used to compute the omega plots may be selected as any value in the observed total concentration range [21].

For the 'reduced' weight-average molecular weight $\overline{M}_w/M_1$, (r) vs. $\bar{c}(r)$ plots the monomer molecular weight M_1 values for bovine insulin, 5734 Da; platypus insulin, 5865 and HB10D platypus insulin, 5843 Da, previously calculated with the PEPTIDESORT program from the Genetics Computer Group (GCG) Sequence Analysis Software Package Version 7.1 [27], were used in these calculations.

All other equations referred to in this section and used for the calculations were installed in the application program GraphPadPRISM Version 1.0 from Graphpad Software (San Diego, CA, USA). The nonlinear regression algorithm of this application program was employed in the curve fitting procedures.

4. Results

4.1. The dependence of 'reduced' weight-average molecular weight on total insulin concentration

In the sedimentation equilibrium experiments conducted with zinc-free insulin, the ionic strength was fixed at 0.1 M and the temperature 25°C to permit the correlation of results over a range of total insulin concentration at 0–1.5 g l^{-1}. This correlation was achieved in several experiments by employing different loading concentrations and angular velocities. In this range of protein concentrations, it is assumed that the samples obey the Beer–Lambert Law where absorbance and concentration are proportional. The zinc-free buffer employed in all cases was 20 mM Tris, 18 mM HCl, 79 mM NaCl, 1 mM EDTA, pH 7.0, ionic strength, I, 0.1 M.

The weight-average molecular weight $\overline{M}_w(r)$ and the omega values as a function of total concentration were calculated for the three insulins using the RALSTON computer program (G.B. Ralston, Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia). An increase in $\overline{M}_w(r)$ with increasing total concentration can give the first insight into the nature of the association.

Plots of $\overline{M}_w/M_1(r)$ (the 'reduced' weight-average molecular weight) as a function of total protein concentration $\bar{c}(r)$ were constructed for all the insulins. All the results from all experiments for bovine insulin (Fig. 1a), regardless of the loading concentrations and angular velocity, were coincident. The same was found for the experiments with platypus (Fig. 1b) and HB10D platypus insulins (Fig. 1c). This supports the observation that all the insulin samples were homogeneous. It is clear from the above plots for bovine and platypus insulin that the

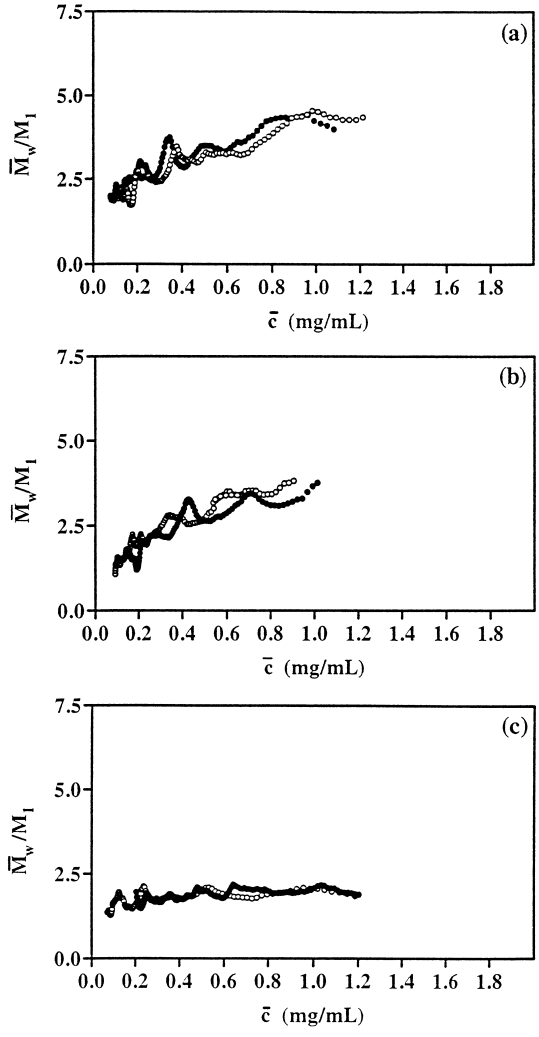


Fig. 1. (a) The overall extent association of zinc-free bovine insulin (pH 7.0; $I = 0.10$ M; $T = 25$°C) reflected in plots of the concentration dependence of the 'reduced' weight-average molecular weight. (b) The overall extent of association of zinc-free chemically synthesised platypus insulin reflected in plots of the concentration dependence of the 'reduced' weight-average molecular weight. (c) The overall extent of association of zinc-free chemically synthesised HB10D platypus insulin reflected in plots of the concentration dependence of the 'reduced' weight-average molecular weight.

$\overline{M}_w/M_1(r)$ increases with increasing total protein concentration, an indication of self-association. The plots for HB10D platypus insulin (Fig. 1c) also indicate self-association but to a lesser extent. In fact, this value does not increase above two at a total concentration of 0.5 g l^{-1} and higher. This is an indication that the major associated species of this analogue is dimer over the concentration range and conditions used.

4.2. Omega analysis

Omega values $\Omega(r)$, as a function of total concentration $\bar{c}(r)$, using Eqs. (1a), (1b) and (1c) were calculated for the three insulins using the RALSTON computer program. All data sets were used for the calculation of $\overline{M}_w(r)$ as a function of total concentration using Eqs. (6a), (6b) and (6c). Different reference concentrations within the concentration distribution of each insulin, namely 0.4, 0.45, 0.5 and 0.55 g l^{-1}, respectively, were used in the omega analysis.

Plots of $\Omega(r)$ vs. $\bar{c}(r)$, for each reference concentration, were constructed for the three insulins. For each specific reference concentration (0.4, 0.45, 0.5 and 0.55 g l^{-1}), $\Omega(r)$ vs. $\bar{c}(r)$ plots were coincident for a particular insulin. Thus, over the common concentration range, the data for all insulins showed acceptable overlap, indicating the attainment of both chemical and sedimentation equilibrium. This good overlap was the same with the different preparations of chemically synthesised platypus insulin.

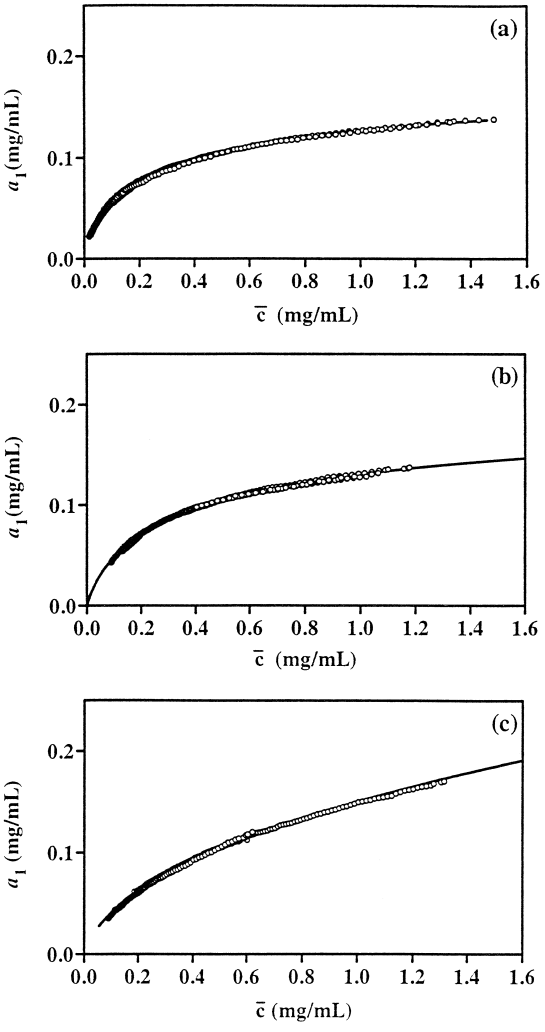
The coincident plot of $\Omega(r)$ vs. $\bar{c}(r)$ for bovine insulin at this reference concentration was extrapolated to yield a value of Ω^0 at $\bar{c} = 0$. It was difficult to determine accurately the value of Ω^0 at $\bar{c} = 0$, as the data covering the lowest $\bar{c}$ values seemed unreliable and were thus disregarded. Therefore, a range of possible Ω^0 at $\bar{c} = 0$ values between 0.18 and 0.22 were tested and used in the fitting procedure. The value of Ω^0 at $\bar{c} = 0$ of 0.21 gave the best fit according to the R^2 values obtained. It was therefore decided to use this extrapolated value as a starting point for all three insulins in the calculation of a_1 as a function of total protein concentration, using Eq. (3). By definition, Eq. (1b) can be rewritten as:

$$\Omega(r) = 1 \text{ at } \bar{c}(r),$$

and constructs of $\overline{M}_w/M_1(r)$ vs. $\bar{c}(r)$ curves via Eqs. (6a), (6b) and (6c), using the association constants K_A and K_B, derived from the omega analysis, return the origin of the curves at infinite dilution at unity (at infinite dilution $\overline{M}_w = M_1$ and $M_1/M_1 = 1$). Although slight errors in the extrapolation of the omega function would not necessarily be detected

[28], it was found that the chosen Ω^0 value of 0.21 (reference concentration 0.5 g l^{-1}) gave the best fit of the experimental data to the IDI model equation for all three insulins.

Since the system may be considered to a good approximation as being thermodynamically ideal in this low concentration range (the activity coefficient y_i being close to unity), corresponding values of m_1 ($m_1 = a_1/M_1$), and $\bar{c}$ were calculated from the $a_1(r)$ vs. $\bar{c}(r)$ curve. Estimates of K_A and K_B values, were obtained by fitting the experimental points of m_1 and its corresponding $\bar{c}$ values, using the nonlinear regression method, to Eq. (5) [23] that describes the self-association of zinc-free insulin (IDI model).



These experimental points, ($a_1(r)$ with the corresponding $\bar{c}(r)$ values), together with the fitted curves (solid lines) that extrapolated smoothly to the origin as required, are shown for all three insulins in Fig. 2a–c.

The results of the fitting procedure converged and two consecutive iterations changed the sum-of-squares by less than 0.01%. A graph of the residuals (the distance of a point from the curve) in all cases showed that the deviation of the experimental points from the best fit curve were within experimental error. The R^2 values, a measurement of goodness of fit, were all greater than 0.99 in all cases, indicating excellent fits within experimental precision.

The values of K_A and K_B were used to calculate the smooth monotonic dependence of $\overline{M}_w/M_1(r)$ on $\bar{c}(r)$ using Eqs. (6a), (6b) and (6c). These calculated

Fig. 2. (a) A plot of the thermodynamic activity of monomer, a_1 vs. the total weight concentration from the sedimentation equilibrium experiments conducted with zinc-free bovine insulin. Eq. (3) was used to calculate the experimental points. The solid line shows the final result of the fitting procedure of experimental m_1 values with nonlinear regression to Eq. (5) (describing the IDI model of self-association) and a_1 values were back calculated with $m_1 = a_1/M_1$. The solid curve was calculated using Eq. (5) with estimates values $K_A = 25.0\times10^3$ M^{-1} and $K_B = 8.0\times10^3$ M^{-1} and shows the final result of the curve fitting procedure used to obtain these estimates. (b) A plot of the thermodynamic activity of monomer, a_1 vs. the total weight concentration from the sedimentation equilibrium experiments conducted with zinc-free chemically synthesised platypus. Eq. (3) was used to calculate the experimental points. The solid line shows the final result of the fitting procedure of experimental m_1 values with nonlinear regression to Eq. (5) (describing the IDI model of self-association) and a_1 values were back calculated with $m_1 = a_1/M_1$. The solid curve was calculated using Eq. (5) with estimates values $K_A = 47.0\times10^3$ M^{-1} and $K_B = 3.4\times10^3$ M^{-1} and shows the final result of the curve fitting procedure used to obtain these estimates. (c) A plot of the thermodynamic activity of monomer, a_1 vs. the total weight concentration from the sedimentation equilibrium experiments conducted with zinc-free chemically synthesised HB10D platypus. Eq. (3) was used to calculate the experimental points. The solid line shows the final result of the fitting procedure of experimental m_1 values with nonlinear regression to Eq. (5) (describing the IDI model of self-association) and a_1 values were back calculated with $m_1 = a_1/M_1$. The solid curve was calculated using Eq. (5) with estimates values $K_A = 103.9\times10^3$ M^{-1} and $K_B = 0.69\times10^3$ M^{-1} and shows the final result of the curve fitting procedure used to obtain these estimates.

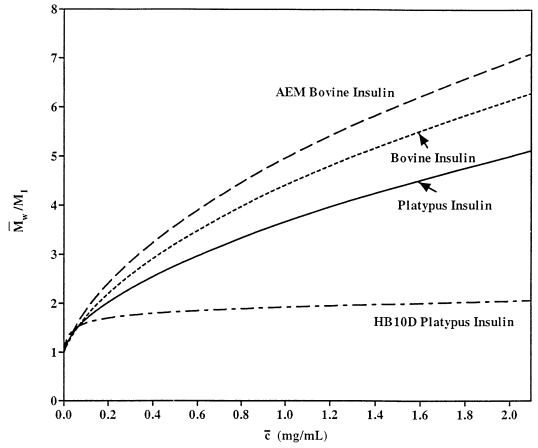


Fig. 3. A comparison of the overall extent of association of zinc-free bovine, chemically synthesised platypus and HB10D platypus insulin and bovine insulin (AEM) from work by Mark et al. [1] as reflected in plots of the concentration dependence of the 'reduced' weight-average molecular weight. The K_A and K_B values, for the three insulins in this study, and values obtained by Mark et al. [1] for the insulin were used to calculate the smooth monotonic dependence of $\overline{M}_w / M_1$ on $\bar{c}$ using Eqs. (6a), (6b) and (6c).

solid curves for each insulin are displayed in Fig. 3. With all three insulins, there was a good fit between the calculated curves and the experimental results.

Comparison of the overall extent of association of zinc-free bovine and platypus insulins at neutral pH, as reflected in the experimental plots of the concentration dependence of the 'reduced' weight-average molecular weight (Fig. 1) and the calculated curves using Eqs. (6a), (6b) and (6c) (Fig. 3), indicates some reduction in the extent of self-association of platypus insulin compared to bovine insulin in the same concentration range. The same comparison for bovine and HB10D platypus insulins reveals a dramatic reduction in the extent of self-association (oligomeric state, approximately two) of the HB10D platypus insulin compared to bovine insulin in the same concentration range. To put these results in perspective, they are also compared in Fig. 3 with the values obtained for bovine insulin by Mark and Jeffrey [16] whose K_A and K_B values were derived from analysis of results over a bigger concentration range, when using the model E ultracentrifuge and a different mode of analysis.

A comparison of published results [1,13,29–31] and those presented here of the state of the association of different insulins demonstrates the variation in values when different methods of sedimentation equilibrium data collection are used. The solution conditions varied only slightly. For instance, the $\overline{M}_w/M_1(r)$ of bovine insulin at 1 mg ml^{-1} varies from 5 [1] to 4.2 [30] compared to 4.5 found in this study and at 2 mg ml^{-1} from 7 [1] to 5 [30] and 6.5 (this study).

4.3. Evaluation of the weight-fractions of all species up to and including hexamer as a function of total insulin concentration

According to the IDI model of self-association of insulin in the concentration range 0–2 g l^{-1} (0–0.33 mM) in a zinc-free neutral pH solution, bovine insulin (and human insulin) exists as a complex distribution of monomers, dimers, trimers, tetramers, pentamers, hexamers and higher order polymers. This relative distribution is dependent on the total concentration of insulin and can be analysed in terms of the weight-fraction for a particular species as a function of total concentration.

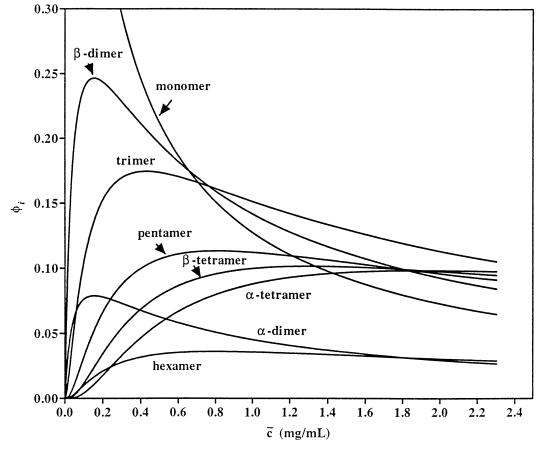


Fig. 4. The weight-fractions (ϕ_i) for all species up to and including ($i = 6$) present in a solution of zinc-free bovine insulin as a function of total concentration is shown. The values for the weight-fractions were calculated using Eqs. (7a), (7b) and (7c) assuming values for K_A and K_B. The lines labelled α-dimer and α-tetramer refer to the species of dimer and tetramer with two exposed α-faces, whereas the lines labelled β-dimer and β-tetramer refer to the species of dimer and tetramer with two exposed β-faces.

The values for weight-fractions of all species up to and including hexamer ($i = 6$) present in solutions of zinc-free insulin (pH 7.0; $I = 0.1$ M; $T = 25$°C) as a function of total concentration were calculated, using Eqs. (7a), (7b) and (7c), and are displayed graphically in Figs. 4–6.

The graphs of both bovine insulin (Fig. 4) and platypus insulin (Fig. 5) illustrate the presence of monomers, α- and β-dimers, trimers, α- and β-tetramers, pentamers and hexamers in the specified concentration range, albeit in different amounts as related by the difference in K_A and K_B values. In contrast, from the weight distribution of all species of HB10D platypus insulin as a function of total concentration (Fig. 6), it is seen that under the conditions used and in the concentration range of 0–2 g l^{-1}, only monomers and β-dimers exist in solution. The tetramers shown are artefactual, generated by the use of a finite (69 M^{-1}) but not significant value for β dimer formation.

The concentration range of 0–2 g l^{-1} for insulin samples was used in the analysis because the changes in weight-fractions of the different polymeric (oligomeric) species are most significant in this range. Comparison of Figs. 4–6 reveals that there is little difference between the weight-fractions of the monomers of the various insulins at any given concentration. However, these graphs, based on the K_A and K_B values for each insulin, clearly illustrate that the weight-fraction of dimers for HB10D platypus insulin is far greater in magnitude than bovine and platypus insulins. At a total concentration of 0.5 g l^{-1} and greater, the weight-fraction value for dimer is 0.8, i.e., 80% of all species by weight. This is the quantitative reflection of the dramatic reduction in $\overline{M}_w/M_1(r)$ of HB10D platypus insulin compared with bovine insulin observed in the primary data (Fig. 1). This supports observations of others that a similar HB10D human insulin exists mainly as dimers under similar solution conditions [11,13]. In the case of platypus insulin, K_A is higher and K_B lower than bovine insulin and this difference leads to a greater weight-fraction of dimers with a concomitant reduction of other species for platypus insulin. This shows up in the slightly reduced $\overline{M}_w/M_1(r)$ vs. $\bar{c}(r)$ dependence of platypus insulin compared to bovine insulin shown in Fig. 3.

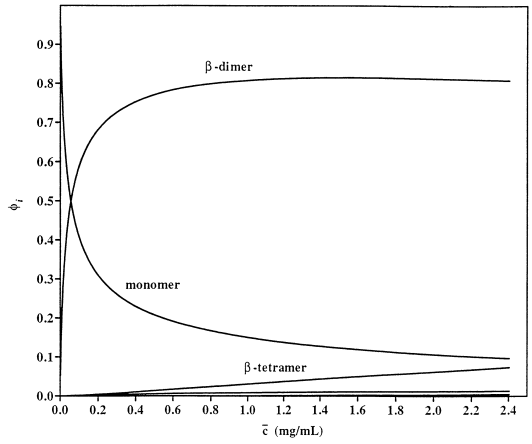


Fig. 6. The weight-fractions (ϕ_i) for all species up to and including ($i = 6$) present in a solution of zinc-free chemically synthesised HB10D platypus insulin as a function of total concentration is shown. The values for the weight-fractions were calculated using Eqs. (7a), (7b) and (7c) assuming values for K_A and K_B. The lines labelled α-dimer and α-tetramer refer to the species of dimer and tetramer with two exposed α-faces, whereas the lines labelled β-dimer and β-tetramer refer to the species of dimer and tetramer with two exposed β-faces.

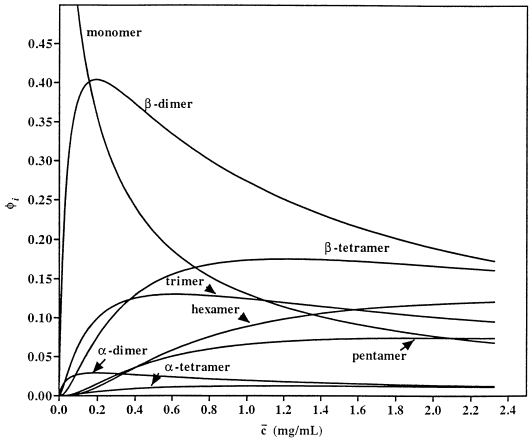


Fig. 5. The weight-fractions (ϕ_i) for all species up to and including ($i = 6$) present in a solution of zinc-free chemically synthesised platypus insulin as a function of total concentration is shown. The values for the weight-fractions were calculated using Eqs. (7a), (7b) and (7c) assuming values for K_A and K_B. The lines labelled α-dimer and α-tetramer refer to the species of dimer and tetramer with two exposed α-faces, whereas the lines labelled β-dimer and β-tetramer refer to the species of dimer and tetramer with two exposed β-faces.

Whereas bovine and platypus insulins form both α- and β-dimers, in HB10D platypus insulin, only β-dimers exist in this concentration range. Compared to other insulins, the K_A value for HB10D platypus insulin is huge (indicating a strong α–α interaction) and the K_B value so low as to indicate that the β–β interaction has been essentially destroyed. In bovine and platypus insulin, the β-dimers are the dominant dimer in solution in these conditions, with the weight-fraction of β-dimers of platypus insulin being higher than that in bovine insulin.

It can be seen in Figs. 4 and 5 that there is hardly a difference in weight-fractions of hexamers between bovine insulin and platypus insulin in contrast to the dimers. As expected there are no HB10D hexamers. These figures are a graphic demonstration of how the relative values of K_A and K_B for a particular insulin can determine the identity and relative amount of a specific polymeric (oligomeric) species in solution at a specific total concentration.

Weight-fractions can be calculated in the same way for trimers, tetramers, pentamers and so on but such calculations merely reinforce the trends already illustrated. That is, that the fractions of species above dimer are lower for platypus insulin than for bovine insulin and that virtually no aggregated species above dimer exist for HB10D platypus insulin.

5. Discussion

Fitting the experimental data to a mathematical equation describing the IDI model of self-association of insulin was excellent as interpreted from the results of the nonlinear regression analysis, namely R^2 values, standard errors and 95% confidence intervals. A visual inspection of the fitted curve and experimental data (Fig. 2) for all the different insulins support the goodness of fit parameters in all cases. The results obtained previously by Mark et al. [1] show that in the environment under discussion, the values of the activity coefficients, y_i, differ very little from unity, even for higher polymeric species. Bovine, platypus and HB10D platypus insulins are overwhelmingly monomeric at low concentrations as is shown graphically in Figs. 4–6. This can be expected since in humans, the plasma insulin concentration range is 0.7–3 ng ml^{-1} [32] and in vitro binding and biological activity studies in cells maximum stimulation occurs at 10 ng ml^{-1} [33]. Insulin solutions containing 10 ng ml^{-1} of insulin would comprise 99.987% monomer by weight using Eqs. (7a), (7b) and (7c). Apart from the existence of zinc hexamer crystals in the β-granules of the pancreas [22], in a biological context, no function for the self-association of insulin has been found in vivo [2,34].

The sedimentation equilibrium analysis of HB10D platypus insulin demonstrate conclusively that it is a monomer–dimer equilibrium in a zinc-free neutral pH environment and is similar to HB10D human insulin [13]. The successful fitting of the sedimentation equilibrium data of this dimeric insulin analogue, HB10D platypus insulin, to the IDI model of insulin self-association reinforces the validity of this model. The substitution of an aspartic acid for a histidine residue in the B chain has produced exactly the result that the model would predict, namely the elimination of the second route of dimer formation and hence, the removal of the possibility of forming aggregates higher than dimers.

It can be concluded that the IDI model for the self-association of insulin is supported by data in the present study for a specified solution environment (zinc-free, neutral pH, $I = 0.1$, $T = 25°C$) and that previously obtained by others [16,1].

For all insulins, the association constants K_A and K_B calculated from the omega analysis produce calculated values of $\overline{M}_w/M_1(r)$ in good agreement with the experimental data points. The K_A derived for bovine insulin in this study, 25.0×10^3 M^{-1}, compares well with the value, 27.4×10^3 M^{-1}, obtained by Mark and Jeffrey [16] in their sedimentation equilibrium study (ideal treatment) under identical solution conditions. The value for K_B, 8.0×10^3 M^{-1}, in this study is, however, slightly lower than the value of 11.8×10^3 M^{-1}, derived by Mark and Jeffrey [16]. This difference may rise from the fact that these authors had to fit data over a much wider concentration range (0.1–3.3 g l^{-1}) gathered with the Rayleigh interference optical system than that (0–1.5 g l^{-1}) used in the present study and to include thermodynamic non-ideality. The overall good agreement in two completely independent studies gives confidence in the results of the analysis of the platypus insulin samples and is also a good

indication of the global error in the association constants resulting from this kind of analysis. Thus, we would say that the best estimate of K_A is $26 \pm 1 \times 10^3$ M^{-1} and K_B is $10 \pm 2 \times 10^3 M^{-1}$. It should be noted that at neutral pH, insulin does not dissolve beyond 4 g l^{-1} [1].

The overall extent of the self-association of bovine, platypus and HB10D platypus insulin differ markedly as is illustrated in Fig. 3 and is governed by the relative values of association K_A and K_B. In all instances, self-association occurs as is illustrated by the dependence of 'reduced' weight-average molecular weight $(\overline{M}_w/M_1)(r)$ of insulin on the total concentration. Even HB10D platypus insulin self-associates, although its highest association state is two in these solution conditions.

From the comparison of the overall extent of association of zinc-free bovine and platypus insulins at neutral pH as reflected in plots of the concentration dependence of the 'reduced' weight-average molecular weight $(\overline{M}_w/M_1)(r)$ vs. $\bar{c}(r)$ (Fig. 3), it can be reasonably concluded that the extent of the self-association of platypus insulin is less than that for bovine insulin. This reduction in self-association is not very large but it is considered to be significant in the light of the estimation of the magnitude of the errors as assessed from the comparison of the two studies of bovine insulin referred to above. Assuming experimental conditions are identical and sample preparations homogeneous the difference in the extent of self-association can be attributed to the different amino-acid residues in the interaction regions (monomer–monomer interface and dimer–dimer interface) of monomeric insulin. The biological activity of the two insulins has been found to be identical (submitted for publication). The K_A value of platypus insulin $(47.0 \times 10^3\ M^{-1})$ is nearly two fold higher in magnitude than that of bovine insulin $(26.0 \times 10^3\ M^{-1})$. The changes in amino-acid residues from bovine insulin to platypus insulin in the monomer–monomer interface (α–α interaction region or OP interaction region in the crystal) are B25 Phe → Tyr and B27 Thr → Ile, respectively. Comparing the properties of the residues [19] and according to previous studies [35], the B25 Phe → Tyr is not expected to have an effect on the extent of monomer–monomer interaction in this region or its receptor binding capabilities. On the other hand, the B27 Thr → Ile substitution introduces a more hydrophobic amino-acid residue in the monomer–monomer interface (α–α interaction region) and has the potential to increase the extent of this self-association as hydrophobic interactions are considered to be the dominant force that govern this interaction as shown [22,36]. Mark et al. [1], in their sedimentation equilibrium studies of the self-association of bovine insulin over a wide pH range and at different ionic strengths and temperatures at pH 7.0, have found that the value of K_A (which in all experimental environments is greater than K_B) increases with increased temperature and increasing ionic strength; both characteristics of interactions which are predominantly hydrophobic in nature [37]. The relatively small dependence of K_A on pH (in comparison to K_B) at fixed ionic strength and temperature is consistent with the above finding for the nature of the α–α interaction region [1]. It is noted that an increase in temperature resulted in an increase of the K_A value and a decrease of the K_B value when analysing the self-association pattern of bovine insulin as obtained using sedimentation equilibrium and the IDI model of self-association [1]. It would be interesting to investigate the influence of temperature on the self-association behaviour of platypus insulin since the platypus has a body temperature of only 32°C. From such a study, some desirable properties may come to light that may be used in the design of therapeutic insulins.

The K_B value, representing the dimer–dimer interface (β–β interaction region or OQ interaction region in the crystal) according to the IDI model, of platypus insulin $(3.4 \times 10^3\ M^{-1})$ is about three times smaller than that of bovine insulin $(10 \times 10^3\ M^{-1})$. The changes in amino-acid residues from bovine to platypus insulin in the dimer–dimer interface are B2 Val → Pro and A13 Leu → Met. When comparing the properties of the residues [19], it is seen that neither substitution would be expected to alter the overall hydrophobic nature of this region. The substitution of B2 Val → Pro, however, has the potential of having an effect on the spatial arrangement of residues in its region as Pro is known to be a breaker of secondary structure [38]. Also, Pro is occasionally found to be the second residue in N-terminal capping boxes of helices [39]. B1-8 in insulin is normally in the extended β-conformation in insulin and a Pro in

the B2 position may give this a more helical character. These properties of Pro in the B2 position in platypus insulin may have an influence on the overall local arrangement of the amino-acid side chains in the dimer–dimer interface and thus translate into a reduced β–β interaction that is reflected in the sedimentation equilibrium experiments on the self-association of platypus insulin. In addition, the hydrophobicity (the value of free energies of transfer) calculated in kcal mole^{-1} for Pro is slightly lower than that for Val [38]. The A13 Leu → Met substitution introduces a sulfur containing amino acid, slightly less hydrophobic than Leu [38], into this region which in turn may effect the spatial arrangement of its neighbouring residues. Although the sulfur may have some H-bonding capability, the major role of methionine is to provide a very flexible hydrophobic side chain [38]. It was indeed found, when using molecular modelling [19], that the spatial arrangement of residues A17 Glu, A13 Met and A14 Tyr in the dimer–dimer interface is very different in platypus insulin. It is possible that this observation could translate into a change (reduction) in the extent of the self-interaction in the dimer–dimer interaction region and which indeed was found.

X-ray analysis of mammalian insulins suggests that the B10 His residue is important for the formation of zinc hexamers [22]. This residue also participates in the hexamer contact (so called dimer–dimer interface) of non-zinc hexamers and thus forms part of the residues involved in the β–β interaction of insulin (OQ interaction in the crystal structure). It must be pointed out, however, that it is by no means certain that the groups involved in the OQ interaction in the crystal [22] are necessarily the same ones involved in the associated states of zinc-free insulin in free solution [16].

The corresponding HB10D human insulin analogue was synthesised and shown to exhibit enhanced bioactivity and receptor binding affinity [20]. Its self-association was explored by the methods of osmometry [11] and limited sedimentation equilibrium [13] and found to be dimeric in the solution conditions and in the concentration range studied. The latter concluded from their 2D NMR studies on this analogue that it is structurally conservative, in other words, no significant structural changes occur upon the substitution at the B10 position. The HB10D platypus insulin analogue also shows a dramatic reduction in the self-association as demonstrated in Fig. 1; which indicates that the self-association state does not increase beyond two. The particular approach in the present work is to analyse the self-association pattern of native and modified insulins by fitting sedimentation equilibrium data to a specific, physically appropriate model of self-association and calculating the association constants. As demonstrated below, this allows changes in self-association patterns brought about by amino-acid substitutions in specific positions to be demonstrated convincingly and to be expressed in quantitative terms.

Although the background sequences between human and platypus insulins are different, it can reasonably be assumed that this does not contribute in a major way to the reduction of self-association as the difference in the extent of the self-association of bovine and platypus is not very marked. In the HB10D platypus insulin, one amino-acid residue change in the dimer–dimer interface has been introduced that, it was predicted, would affect significantly the β–β interaction under these conditions. This hypothesis can be supported by analysing the self-association pattern of this analogue.

The K_A value (100×10^3 M^{-1}) that represents the α–α interaction region (monomer–monomer interface) according to the IDI model of HB10D platypus insulin, is almost four times higher than that of bovine insulin (26.0×10^3 M^{-1}). In contrast, the evaluated K_B value (0.07×10^3 M^{-1}), that represents the β–β interaction region (dimer–dimer interface) according to the IDI model, of HB10D platypus insulin, is effectively zero (bovine insulin 10.0 $\pm 2 \times 10^3$ M^{-1}). It may be concluded that this huge reduction in K_B value virtually abolishes the β–β interaction involving the dimer–dimer interface. The increase in the K_A value for HB10D platypus insulin could be explained as follows: with the abolishment of the β–β interaction, and thus removal of this previously (in the native sequence insulins) competing self-association event, the availability for association via the α–α interaction region increases. In other words, the α–α interaction is stronger in the absence of a competing β–β interaction. These quantitative findings demonstrate nicely that quantitative analysis of sedimentation equilibrium data employing a specific model allows for meaningful cor-

relations to be made between changes at the molecular level and their subsequent expression in changes in self-association patterns.

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