Biophysical Chemistry 27 (1987) 103–117
Elsevier

BPC 01161

The self-association of zinc-free bovine insulin

A single model based on interactions in the crystal that describes the association pattern in solution at pH 2, 7 and 10

Alan E. Mark [a], Lawrence W. Nichol [b] and Peter D. Jeffrey [a]

[a] *Protein Chemistry Group, The John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601 and* [b] *Office of the Vice-Chancellor, University of New England, Armidale, N.S.W. 2351, Australia*

Received 22 December 1986
Accepted 24 February 1987

Insulin; Insulin association pattern; Indefinite self-association; Sedimentation equilibrium

Sedimentation equilibrium studies are used to establish that a new pattern for the self-association of zinc-free insulin in solution is applicable over a wide range of conditions of pH, ionic strength and temperature. In this pattern, which is based on information from the existing literature on the X-ray crystal structure of insulin, the insulin monomer is viewed as having two distinct faces both capable of self-interaction. Sedimentation equilibrium experiments were analysed using expressions formulated for this association pattern that describe the dependence of weight average molecular weight and monomer concentration on total protein concentration. It has thereby been possible to obtain values for the two association constants which govern the system for each set of conditions studied, due allowance having been made for composition dependent non-ideality effects. Furthermore, by relating the pH, temperature and ionic strength dependence of the association constants with properties of various amino acid residues on the surface of the insulin monomer, it has also been possible to assign tentatively each constant to a particular reaction domain.

1. Introduction

The pattern of self-association of zinc-free insulin (monomer molecular weight, 5734) has been extensively studied by a wide variety of techniques including NMR [1], circular dichroism [2], sedimentation velocity [3], sedimentation equilibrium [4] and light scattering [5]. Several basic models have been proposed to describe the self-association under various conditions. Jeffrey and Coates [6] working at pH 2.0 proposed a definite association pattern consisting of monomer, dimer, tetramer and hexamer. The same pattern was later shown by Goldman and Carpenter [2] to be consistent with results obtained at pH 8.0. While some workers have proposed that at pH 7.0, the association is definite like that proposed at pH 2.0 and 8.0 [7,8], this does not account for the fact that at pH 7.0 species considerably larger than hexamer are present in solution at concentrations of insulin as low as 2 g/l [9]. In an attempt to account for this observation, Pekar and Frank [9] proposed a combined definite and indefinite association pattern in which monomer and dimer were in equilibrium with hexamer which then isodesmically self-associated to give polymers of the hexamer species. This combination of definite and indefinite association patterns differs from that mentioned earlier, in that the concentrations of all species intermediate between dimer and hexamer have been taken as negligible. The self-association

Correspondence address: A.E. Mark, Protein Chemistry Group, The John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia.

pattern at pH 7.0 was also investigated by Jeffrey et al. [4]. These workers tested a number of models including that of Pekar and Frank [9] and an isodesmic indefinite self-association. They showed that when non-ideality was taken into consideration the only model capable of fitting the data was one where monomer was in equilibrium with dimer which then acted as the basic protomer for a further isodesmic indefinite self-association. Jeffrey et al. [4] implicitly assumed that only one type of dimer would be formed by self-interaction of one available reaction domain which resulted in a conformational change exposing the second reaction domain and thereby permitted isodesmic self-association of the dimer via self-interaction of this exposed domain. Wollmer et al. [10] were the first, albeit in a semi-qualitative treatment, to suggest that this model was unnecessarily complicated in that the insulin monomer itself was bifunctional. Specifically, the latter workers proposed two types of dimer, successive addition of monomer to each creating both odd- and even-numbered polymers. Later Nichol et al. [11] derived an equation pertinent to this concept and showed that the sedimentation equilibrium results which had been analysed in terms of the previous model could also be described with considerable precision by one involving two types of dimer. It must be stressed however that the results, which were shown to be in accord with the reasonable postulate of a 'head-to-head' and 'tail-to-tail' association governed by two site-binding constants k_α and k_β, referred only to a limited range of total concentration (0–3 g/l) and to a particular environment (pH 7.0, $I = 0.2$, $T = 25\,^\circ$C). Accordingly, while the postulate may be reasonable on the basis of known structural information on insulin, it cannot be claimed to have been tested over a wide range of conditions. This work presents an analysis of the results obtained with solutions of zinc-free insulin over a range of pH, ionic strength and temperature. It addresses the following questions.

(1) Does the fundamental nature of the association pattern change as the pH is varied or is a single pattern operative, despite the variation attributed to the system by different investigators?

(2) Is it correct to confer special stability on the zinc-free hexamer species, as has been done in all work reported except that of Jeffrey et al. [4]?

(3) Is the assumption, shared by all models thus far suggested, valid that the association proceeds in a manner in which certain polymers assume negligible equilibrium concentrations?

(4) Is it possible to correlate empirically observed changes in k_α and k_β with variation in the behaviour of constituent residues in monomer-monomer reaction domains?

The method selected for this study was sedimentation equilibrium, because it is capable of yielding with considerable precision information on the dependence on total concentration of both weight-average molecular weight and of the concentration of the momoner m_1.

2. Materials and methods

2.1. Protein solutions

All experiments were conducted with bovine insulin obtained from Commonwealth Serum Laboratories and designated crystalline and 'single peak'. The following steps were employed in the purification of the sample. First, the protein was dissolved in and exhaustively dialysed against 0.01 M HCl to form the apo-protein free of bound Zn^{2+} [4]. The last stages of the dialysis were performed against a solution of 0.01 M HCl and 0.1 M NaCl. The second step involved subjecting the dialysed solution to gel filtration on Sephadex G-50 to remove traces of proinsulin. The final step involved adjusting the pH of the solution to 8.1 in accordance with the method of Chance et al. [12] and subjecting the solution to ion-exchange chromatography on a column of DEAE-cellulose to remove any traces of monodesamido-insulin. The electrophoretic homogeneity of purified samples was routinely checked on polyacrylamide gels and samples were stored in the freeze-dried state at $-20\,^\circ$C.

Solutions of insulin were prepared by dissolving the freeze-dried zinc-free insulin powder either directly in the appropriate buffer or (for experiments conducted at pH 7.0) initially in 0.01 M HCl. The compositions of all buffers used in the

Table 1

The composition of buffers employed in sedimentation equilibrium studies on zinc-free insulin

Buffer composition	pH [a]	Ionic strength, I	Density [b] (g/ml)
5.3 mM glycine, 14.7 mM HCl, 85.3 mM NaCl	2.0	0.10	1.0015_7
20 mM Tris, 18 mM HCl, [c] 79 mM NaCl, 1 mM EDTA	7.0	0.10	1.0025_0
20 mM Tris, 18 mM HCl, 29 mM NaCl, 1 mM EDTA	7.0	0.05	0.9995_0
5.3 mM glycine, 3.2 mM NaOH, 95 mM NaCl	10.0	0.10	1.0012_5

[a] All pH values were measured at the temperature of the sedimentation equilibrium experiments.

[b] Densities of buffers were measured with an Anton Paar DMA 02C precision density meter accurate to ±0.00001 g/ml.

[c] In two sedimentation equilibrium experiments conducted at 37°C rather than 25°C the pH of the Tris-HCl buffer was adjusted to pH 7.0 at 37°C by addition of NaOH. The resulting density of the buffer was 0.9977_0 g/ml.

sedimentation equilibrium experiments are given in table 1. Solutions, filtered through 0.22 μm Millipore filters, were adjusted to approximately the desired concentration, and dialysed (Spectrapor type 3, M_w cut-off 3500) for 18–24 h against buffer with several changes of dialysate. Concentrations of solutions were determined spectrophotometrically at 276 nm employing an extinction coefficient of $E^{1\%}_{1cm} = 10.5$ [13]. Dilutions where necessary were performed with dialysate. Initial loading concentrations, $\bar{c}_0$, for the sedimentation equilibrium experiments were checked refractometrically employing either a Brice-Phoenix differential refractometer or by performing a synthetic boundary experiment in the ultracentrifuge using Rayleigh interference optics. The specific refractive index of insulin was taken to be 1.789×10^{-4} g/l [14] which in a 12 mm ultracentrifuge cell leads to the conversion that 3.93 Rayleigh interference fringes corresponds to 1.0 g/l, one fringe being equivalent to a displacement of 287 μm. For simplicity and consistency all concentrations in this work are reported directly on the g/l scale.

2.2. Sedimentation equilibrium experiments

A Spinco model E analytical ultracentrifuge fitted with an electronic speed control was used to conduct all sedimentation equilibrium experiments which were of either short column [15] or Chervenka [16] meniscus depletion design. A Rayleigh interference optical system in conjunction with a symmetrical limiting aperture was used to follow the progress of all experiments, with interferograms being recorded photographically on Kodak IIG plates. Throughout the course of an experiment the temperature of the rotor was controlled to within ±0.1°C of the value quoted in section 3 using the RTIC and refrigeration units of the ultracentrifuge. All experiments were conducted using an aluminium AN-D rotor in conjunction with a 12 mm double-sector cell with a filled-epon centre piece, sapphire windows and teflon gaskets. Experiments were designed so that the total concentration of insulin at the base of the cell was well below the solubility limits of insulin. Selection of the required conditions was based on previously published parameters of the system [6,9,14] and dialysed solutions and their equilibrium dialysates were used in solution and solvent sectors of the cell, respectively [17]. It had been previously shown [14] that 24 h was sufficient to ensure equilibrium had been established. Nevertheless, the attainment of equilibrium was always checked by comparing the interferograms after 20 and 24 h. Final sedimentation equilibrium distributions were recorded as interferograms which were measured using a Nikon model-6C microcomparator according to the method of Richards et al. [15]. The equilibrium distributions were analysed in accordance with the method of Milthorpe et al. [18] to obtain the activity of monomer, a_1, as a function of total concentration, $\bar{c}$.

3. Theory

3.1. The dependence of monomer concentration on total concentration

The model for the self-association of insulin under consideration involves indefinite self-associ-

ation of a bivalent monomer by a combination of like domains, a so-called head-to-head and tail-to-tail association. In more formal terms, the monomer possesses two independent non-identical self-association sites, designated α and β, both capable of self-interaction. Two types of dimer are formed, one involving an α-α interaction, leaving two β-sites exposed and governed by an association constant k_α; the other involving β-β interaction, leaving two α-sites exposed and governed by an association constant k_β. 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 a β-site at the other. This distinction proved helpful in that Nichol et al. [11] were able to show that:

$$c_i = iM_1(4k_\alpha k_\beta)^{(i-1)/2} m_1^i; \qquad i \text{ odd} \tag{1a}$$

$$c_j = jM_1(k_\alpha + k_\beta)(4k_\alpha k_\beta)^{(j-2)/2} m_1^j; \qquad j \text{ even} \tag{1b}$$

Thus,

$$\bar{c} = M_1 m_1 \left[\sum_{i=1}^{i=\infty} i(4k_\alpha k_\beta)^{(i-1)/2} m_1^{i-1} + \sum_{j=2}^{j=\infty} j(k_\alpha + k_\beta)(4k_\alpha k_\beta)^{(j-2)/2} m_1^{j-1} \right] \tag{2}$$

which on summation yielded:

$$\bar{c} = M_1 m_1 \left[\frac{(1 + 2k_\alpha m_1)(1 + 2k_\beta m_1)}{(1 - 4k_\alpha k_\beta^2 m_1^2)} \right] \tag{3}$$

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.

Eq. 3 mathematically describes the composition of a solution comprising an infinite array of species in solution of basically different types (odd- and even-numbered polymers). However, unlike all other patterns of associations previously treated, no expression is available from the literature for the weight-average molecular weight.

3.2. Dependence of 'reduced' weight-average molecular weight ($\bar{M}_w/M_1$) on total concentration

Combination of eqs. 1a and 1b with the expression for the weight-average molecular weight, $\bar{M}_w$,

$$\bar{M}_w = \sum_{i=1}^{i=n} M_i c_i / \sum_{i=1}^{i=n} c_i$$

where $n = \infty$, yields:

$$\frac{\bar{M}_w}{M_1} = m_1 M_1 \left[\sum_{i=1}^{i=\infty} i^2 (4k_\alpha k_\beta)^{(i-1)/2} m_1^{i-1} + \sum_{j=2}^{j=\infty} j^2 (k_\alpha + k_\beta)(4k_\alpha k_\beta)^{(j-2)/2} m_1^{j-1} \right] / \bar{c} \tag{4}$$

The first sum in the numerator may be expanded as, $1 + 9(4k_\alpha k_\beta)m_1^2 + 25(4k_\alpha k_\beta)^2 m_1^4 + 49(4k_\alpha k_\beta)^3 m_1^6 + \ldots + \infty$ which is in the general form of $1 + 3^2x + 5^2x^2 + 7^2x^3 + \ldots + \infty$ where $x = 4k_\alpha k_\beta m_1^2$. When $|x| < 1$ this series converges to [19] $(1 + 6x + x^2)/(1 - x)^3$. Thus:

$$\sum_{i=1}^{i=\infty} i^2 (4k_\alpha k_\beta)^{(i-1)/2} m^{(i-1)} = \frac{1 + 24k_\alpha k_\beta m_1^2 + 16k_\alpha^2 k_\beta^2 m_1^4}{(1 - 4k_\alpha k_\beta m_1^2)^3} \tag{5}$$

The second sum when expanded gives $4(k_\alpha + k_\beta)m_1[1 + 4x + 9x^2 + 16x^3 + \ldots + \infty]$; $x = 4k_\alpha k_\beta m_1^2$ which when $|x| < 1$ also may be written in closed form [19], so that:

$$\sum_{j=2}^{j=\infty} j^2 (k_\alpha + k_\beta)(4k_\alpha k_\beta)^{(j-2)/2} m_1^{j-1} = \frac{4m_1(k_\alpha + k_\beta)[1 + 4k_\alpha k_\beta m_1^2]}{(1 - 4k_\alpha k_\beta m_1^2)^3} \tag{6}$$

Substitution of eqs. 5 and 6 into eq. 4 followed by substantial rearrangement leads to:

$$\frac{\bar{M}_w}{M_1} = \frac{P}{Q} \tag{7a}$$

where

$$P = 16k_\alpha^2k_\beta^2m_1^4 + 16k_\alpha k_\beta(k_\alpha + k_\beta)m_1^3 + 24k_\alpha k_\beta m_1^2 + 4(k_\alpha + k_\beta)m_1 + 1 \quad (7b)$$

$$Q = (1 - 4k_\alpha k_\beta m_1^2)(1 + 2k_\alpha m_1)(1 + 2k_\beta m_1) \quad (7c)$$

When $k_\alpha = k_\beta$, i.e., sites are equivalent, eq. 7 reduces to the same form as the equation for an indefinitely self-associating system governed by a single equilibrium constant, K_I (isodesmic) [20], namely

$$\frac{\overline{M}_w}{M_1} = \frac{1 + K_I m_1}{1 - K_I m_1};\ K_I m_1 < 1 \quad (8)$$

with $2k_\alpha = 2k_\beta = K_I$ as required. From the first derivative of eq. 7 it can be shown that there are no critical points in the allowable range of $0 < 4k_\alpha k_\beta m_1^2 < 1$, nor were any points of inflection evident from numerical examples. Thus, it appears that the simultaneous set of equations, eqs. 3 and 7, describe a smooth monotonically increasing dependence of $(\overline{M}_w/M_1)$ on $\bar{c}$, illustrations of which will be presented later.

3.3. Weight fraction of species

The distribution of species in an indefinitely associating system is usefully formulated in terms of the weight fraction of species as a function of total concentration. No such expression has yet been formulated for the head-to-head and tail-to-tail indefinite self-association pattern pertinent to insulin.

The required expression may be formulated from the combination of eq. 3, the expression for the total weight concentration, and eqs. 1a and 1b, the weight concentrations of each of the odd- and even-numbered species, respectively. The weight fraction, ϕ_i, of each species, i, being given by:

$$\phi_i = \frac{c_i}{\bar{c}} = \frac{iX_i m_1^{(i-1)}(1 - 4k_\alpha k_\beta m_1^2)^2}{(1 + 2k_\alpha m_1)(1 + 2k_\beta m_1)} \quad (9a)$$

where

$$X_i = (4k_\alpha k_\beta)^{(i-1)/2};\qquad i \text{ odd} \quad (9b)$$

$$X_i = (k_\alpha + k_\beta)(4k_\beta k_\beta)^{(i-2)/2};\quad i \text{ even} \quad (9c)$$

By differentiating this expression with respect to $\bar{c}$ it is possible to determine whether the weight fraction of a given species will pass through a maximum. It is noted in regard to this differentation that X_i is a constant independent of $\bar{c}$. The weight fraction of a given species will pass through a maximum when $d\phi_i/d\bar{c} = 0$. It may be readily shown that this is only true when $16(i+1)k_\alpha^2k_\beta^2m_1^4 + 8(i+2)k_\alpha k_\beta(k_\alpha + k_\beta)m_1^3 + 24k_\alpha k_\beta m_1^2 + 2(2 - i)(k_\alpha + k_\beta)m_1 - (i-1) = 0$.

When $i = 1$ the last term disappears and, as there are no changes in sign within the polynomial, by Descartes' rule of sign, there can be no real roots for $d\phi_1/d\bar{c} = 0$. Thus, the monomer weight fraction must decrease monotonically with increasing $\bar{c}$ $(d\phi_1/d\bar{c} < 0)$. For all $i > 1$ there is one change of sign and therefore one positive real root for $d\phi_i/d\bar{c}$. This means that the weight fraction of all species other than monomer will pass through a single maximum as the total concentration of insulin is increased. Further attention will be given to this point later.

3.4. Thermodynamic non-ideality

In this work an approach is adopted which acknowledges that activity coefficients are dependent upon the composition of a solution rather than on total concentration of solute and assesses non-ideality coefficients by statistical mechanics in terms of excluded volumes [21]. In studies conducted on insulin the value of $\bar{c}$, never exceeding 6 g/l, only the first term of the expansion of the logarithm of the activity coefficient of each species need be considered:

$$\ln y_i = \sum_j \alpha_{ij} m_j \quad (10)$$

where each of the subscripts i, j is allowed to span the set of monomeric and polymeric species independently. The set of constant coefficients α_{ij} may be calculated utilizing the expression [21]:

$$\alpha_{ij} = \frac{4\pi N(r_i + r_j)^3}{3} + \frac{Z_i Z_j(1 + \kappa r_i + \kappa r_j)}{2I(1 + \kappa r_i)(1 + \kappa r_j)} - M_j\bar{v}_j \quad (11)$$

where the first term denotes the covolume contri-

bution based on spherical geometry, r_i and r_j being radii of the impenetrable spheres; the second term gives the charge-charge interaction in terms of the net charges, Z_i and Z_j, borne by the spheres, the ionic strength, I, and the Debye inverse-screening length, κ; and the third term expresses the molar volume of species j. Once the values of α_{ij} have been tabulated results may be analysed in terms of eq. 10 by an iterative procedure based on that originally proposed by Nichol and Winzor [22]. In more detail, the known values of a_1 obtained by the Ω [18] method are used to obtain estimates of other a_i from equilibrium constants deduced from analysis of results obtained at low $\bar{c}$. These values of a_i including a_1 are inserted into eq. 10 as first estimates of m_i to obtain first estimates of all y_i. Division of the a_i by the first estimates of y_i gives improved values for m_i appropriate to eq. 10. This procedure is repeated until the values of y_i converge. This procedure may be applied at each $\bar{c}$ for which a_1 values are available and does involve, for an indefinitely self-associating system, truncation of those polymers which are assessed to contribute negligibly to the $\bar{c}$ under consideration. In the final step it is possible to test the appropriateness of the equilibrium constants employed by summing the weight concentration of each species obtained as $c_i = iM_1a_i/y_i$ to enquire whether the predicted total concentration in fact agrees with the experimental total concentration corresponding to the particular a_1 value under consideration. If it does not, it is possible to refine the values of the equilibrium constants, due allowance having been made for the composition dependence of the activity coefficients.

The molecular weight of the monomer of bovine insulin was taken to be 5734 [23] with a partial specific volume of 0.73 ml/g [13]. As in previous studies [4] it was assumed that the same partial specific volume characterized all polymeric states of insulin, the implicit assumption being that no measurable volume change accompanies the self-association of the protein. In accounting for the thermodynamic non-idealilty effects according to eq. 11 it is necessary to have estimates of both the effective Stokes radius of each state of the protein and the net charge borne by it. With respect to the former quantities the following relations pertain [24]:

$$r_i^{\mathrm{H}} = \left(3M_i^{\mathrm{H}}\bar{v}_i^{\mathrm{H}}/4\pi N\right)^{1/3} \tag{12a}$$

$$M_i^{\mathrm{H}} = M_i^{\mathrm{U}}(1+w) \tag{12b}$$

$$\bar{v}_i^{\mathrm{H}} = \left(\bar{v}_i^{\mathrm{U}} + wv_1\right)/(1+w) \tag{12c}$$

where the superscript H denotes the hydrated particle, the superscript U the unhydrated particle, w is the degree of hydration (g solvent per g dry solute) and v_1 is the partial specific volume of the solvent (approximately unity). Combination of eqs. 12b and 12c yields:

$$M_i^{\mathrm{H}}\bar{v}_i^{\mathrm{H}} = M_i^{\mathrm{U}}\left(\bar{c}_i^{\mathrm{U}} + wv_1\right) \approx M_i^{\mathrm{U}}, \tag{13}$$

since w for globular proteins is in the range 0.2–0.3 g/g. It follows then, on the basis of spherical geometry, that a reasonable approximation of the effective Stokes radius of the hydrated polymeric states of insulin may be calculated simply from:

$$r_i^{\mathrm{H}} = \left(3iM_1^{\mathrm{U}}/4\pi N\right)^{1/3} \tag{14}$$

where $i = 1$ denotes the insulin monomer. The net charges borne by the monomer of insulin at the different pH values used in this work were taken from the titration results of Tanford and Epstein [25]. The values were as follows: +5 (pH 2.0), −2 (pH 7.0) and −5.5 (pH 10.0). It was assumed that charge was conserved in each step of the self-association to form higher polymers. The values of κ, the Debye inverse-screening length, appropriate to eq. 11 were calculated from the relationship $\kappa \approx 3.3 \times 10^{-7}\sqrt{I}$ [26]. These parameters and relations permit the ready computation of the non-ideality coefficients a_{ij}, which are then used in the iterative procedure previously described to estimate the activity coefficients y_i of each polymeric state. As is apparent from eq. 10, it is necessary in the calculation of y_i for an indefinitely self-associating system to truncate the system at a tractable and realistic value of i.

In the range of total concentration explored at pH 7.0 it was found by numerical calculation that truncation at $i = 30$ sufficed to account for over 98% of the total concentration. Since, as will be

seen, the extent of association is greater at pH 7.0 than at either pH 2.0 or pH 10.0, the truncation value of $i = 30$ was consistently used in the analysis of all results.

4. Results

4.1. Studies at pH 7.0

Discussion is first directed toward results obtained at neutral pH, a condition which has been utilized in several previous studies to explore the self-association pattern of zinc-free insulin. In this work the ionic strength was held fixed at 0.1 and the temperature at 25 °C to permit a correlation of results over a range of total concentration 0.01–3.3 g/l, which was achieved in three experiments, by employing different loading concentrations and angular velocities as described in fig. 1. This figure presents a plot of the apparent weight-average molecular weight vs. the total concentration found using eq. 15.

$$\Phi \overline{M}_w = \frac{\mathrm{d} \ln \bar{c}}{\mathrm{d}(x^2)} \qquad (15)$$

$$\Phi = (1 - \bar{v}\rho)\omega^2/2RT$$

where R is the gas constant, T the absolute temperature, ω the angular velocity, x the distance from the axis of rotation, ρ the solution density and it has been assumed that $\bar{v}$, the partial specific volume of insulin, is the same for all insulin species.

Values of $[\mathrm{d} \ln \bar{c}/\mathrm{d}(x^2)]$ were obtained by differentiation of a polynomial expression used to fit the $\ln \bar{c}$ vs. (x^2) data. Two points emerge from an inspection of fig. 1. Firstly, in accordance with all previous findings, it is apparent that the apparent weight-average molecular weight increases with increasing concentration to the extent that species with molecular weights greater than that of the hexamer ($M_6 \sim 35\,000$) evidently exist at concentrations as low as 1.3 g/l. Clearly, zinc-free

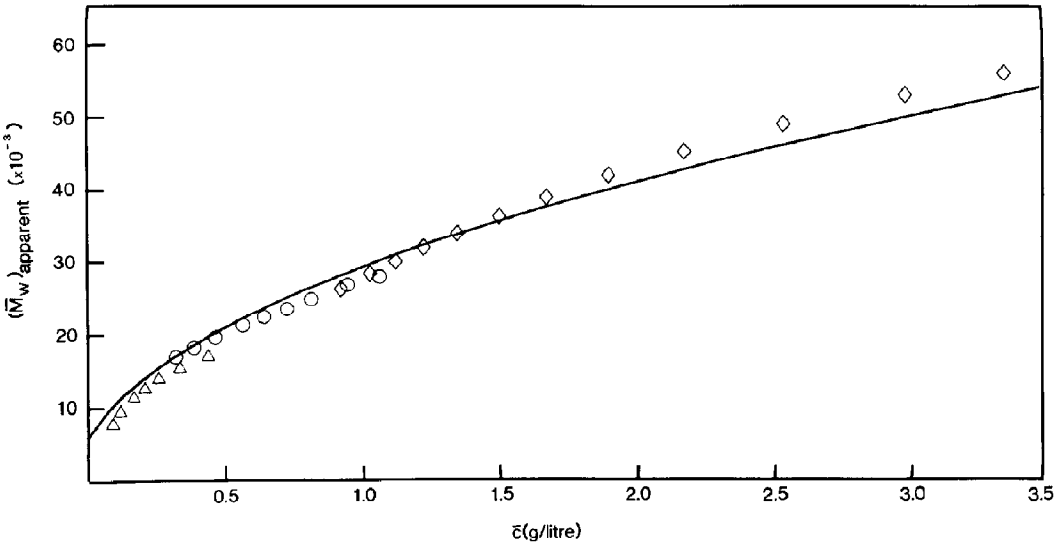

Fig. 1. The dependence of the experimentally determined apparent weight-average molecular weight $(\overline{M}_w)_{apparent}$ on total weight concentration of zinc-free insulin at pH 7.0, $I = 0.10$ and 25 °C. Experimental values were obtained by differentiating sedimentation equilibrium results according to eq. 14. (△) $c_0 = 0.759$ g/l, $\omega = 5028$ rad/s, meniscus depletion; (○) $c_0 = 0.476$ g/l, $\omega = 2724$ rad/s, short column; (◇) $c_0 = 0.905$ g/l, $\omega = 2094$ rad/s, short column. The solid curve was computer using eq. 7 with $k_\alpha = 2.05 \times 10^4$ M^{-1} and $k_\beta = 1.63 \times 10^4$ M^{-1}, values found after allowance had been made for thermodynamic non-ideality effects.

insulin does self-associate to an appreciable extent in the specified environment. Secondly, when account is taken of the uncertainty in $(\overline{M}_w)_{apparent}$ values inherent in differentiation of the fundamental results ($\pm 10\%$), it is reasonable to conclude that the results from the three experiments essentially overlap. This provides further support for the observation made by others at higher ionic strengths that no measurable volume change accompanies the self-association at neutral pH. The solid line in fig. 1 was calculated using eq. 7 and values of k_α and k_β determined after allowing for non-ideality effects. The procedure was as follows. The experimental data in the form of total concentration vs. radial distance were expressed in the form Ω vs. total concentration [18] allowing construction of a plot of the thermodynamic activity of the insulin monomer vs. total concentration. This extrapolated smoothly to the origin. First estimates of $k_\alpha = 2.5 \times 10^4$ M^{-1} and $k_\beta = 1.3 \times 10^4$ M^{-1} were obtained by fitting this plot with eq. 3. Allowance for non-ideality proceeded by calculation of the α_{ij} using eq. 11 and using these values in the iterative procedure, based on eq. 10 outlined earlier. This permitted refinement of the site-binding constants to $k_\alpha = 2.05 \times 10^4$ M^{-1} and $k_\beta = 1.63 \times 10^4$ M^{-1}, which differ little from the values obtained neglecting non-ideality. It is stressed, however, that the allowance for non-ideality assumes greater importance in experimental environments where charge-charge interactions are increased in magnitude. In the course of this analysis it was evident that, in the environment presently under discussion, the values of the activity coefficients differed very little from unity even for the higher polymeric species. For example, at a total concentration of 2.0 g/l y_i for the 12-mer was only 1.112. Likewise, it was confirmed that truncation of the number of species included in the analysis at the 30-mer is certainly sufficient. The appropriateness of the head-to-head and tail-to-tail association pattern to the results obtained

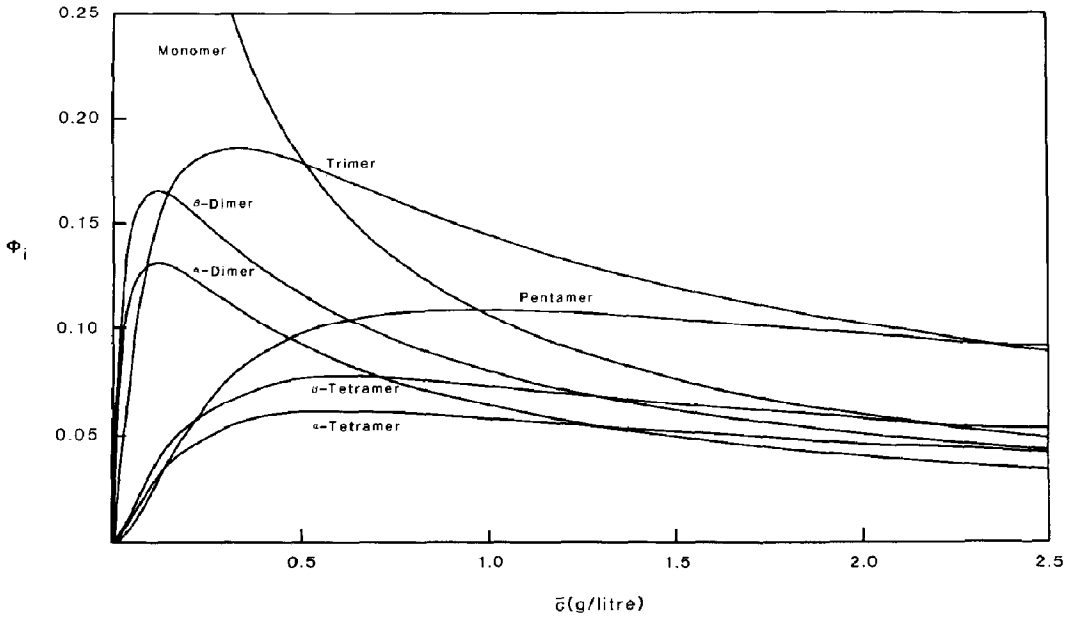

Fig. 2. The weight fractions (ϕ_i) of all species up to and including pentamer ($i = 5$) present in a solution of zinc-free insulin (pH 7.0, $I = 0.1$, $T = 25\,^\circ C$) as a function of total concentration ($\bar{c}$) are shown. The values for the weight fractions were calculated using eq. 9 assuming values for k_α and k_β of 2.05×10^4 and 1.63×10^4 M^{-1}, respectively. 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.

at pH 7.0 is seen by the fit of the calculated solid curve to the experimental points in fig. 1 referring to the differentiated results.

The availability of values of k_α and k_β allows calculation of the weight fraction of the insulin species present in solution under the relevant conditions as a function of total insulin concentration. The results of such calculations are presented in fig. 2 which shows the weight fractions of all species up to and including pentamer ($i = 5$) as a function of total concentration at pH 7.0, $I = 0.1$ and $T = 25\,^\circ$C. It should be noted that in order to account for the two types of dimer and the two types of tetramer separately it is necessary to expand the expression given in eq. 9c. Fig. 2 demonstrates the point made analytically in section 3.3 that with this association model the weight fractions of all species other than monomer pass through a maximum as the total concentration is increased. The graphical presentation allows appreciation of the complex distribution of species within the insulin system in solution. It also stresses the importance of the odd-numbered species, the dominant species by weight, under these particular conditions. This underlines the contrast between this and earlier models for the association of zinc-free insulin, the earlier models taking no account of the possible presence of any odd-numbered species other than monomer.

4.2. Studies at pH 2.0

A major point of interest is whether or not the head-to-head and tail-to-tail association pattern also suffices to describe the results obtained at pH 2.0 a value employed by other workers [6] and one where zinc-free insulin is more soluble than at pH 7.0. An entirely similar procedure to that described earlier was used to obtain a plot of a_1 vs. $\bar{c}$ using the Ω method applied to results obtained in two experiments, details of which are given in fig. 3. The iterative procedure was used to calculate activity coefficients of species at selected concentrations in the experimental range 0.1–5.8 g/l. At pH

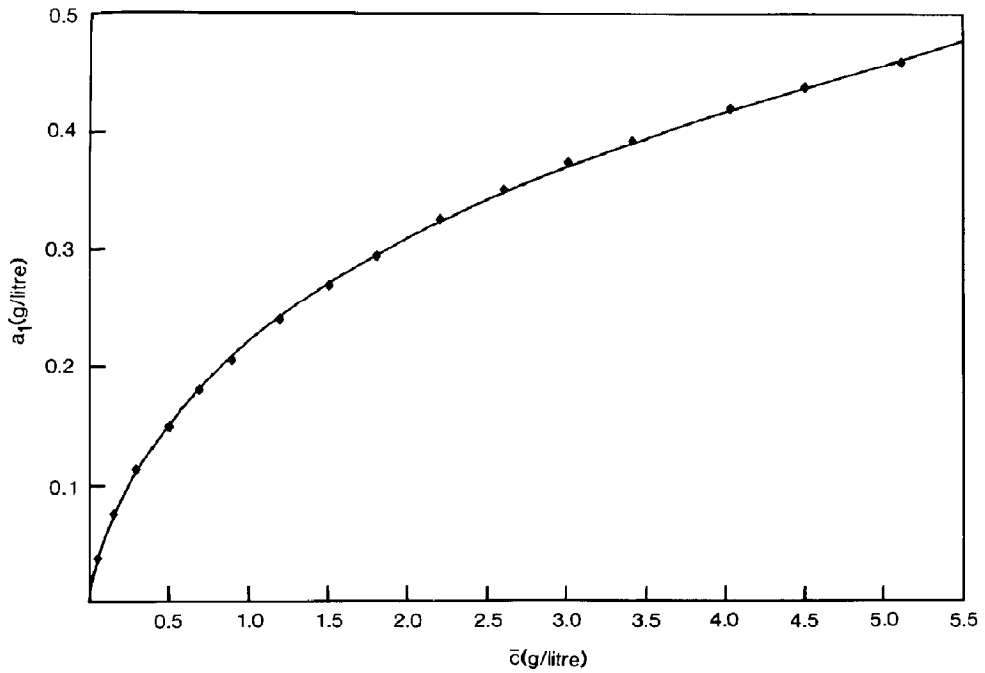

Fig. 3. The result of analysing sedimentation equilibrium results, by the Ω method, obtained at pH 2.0, $I = 0.10$ and 25 °C with zinc-free insulin. The results of two experiments are included: $c_0 = 0.710$ g/l, $\omega = 4609$ rad/s, meniscus depletion; $c_0 = 1.823$ g/l, $\omega = 3143$ rad/s, short column. The solid line utilized refined values of k_α and k_β (3.80×10^4 and 0.03×10^4 M^{-1}, respectively) obtained after allowance had been made for composition-dependent non-ideality effects.

2.0 the charge on the monomer is +5 and thus the charge-charge interaction term in eq. 11 is much larger at pH 2.0 than at pH 7.0. This may be illustrated by comparing the magnitude of the acitivity coefficients, for example, the value $y_{10} = 1.094$ at pH 7.0 with that of $y_{10} = 1.50$ found at the same total concentration of 2.0 g/l at the lower pH. When due allowance was made for composition-dependent non-ideality, the refined values of k_α and k_β which best fitted the results at pH 2.0 were found to be $k_\alpha = 3.8 \times 10^4$ M^{-1} and $k_\beta = 0.03 \times 10^4$ M^{-1}. Fig. 3 examines the adequacy of the postulated association pattern governed by these two site-binding constants. It compares experimental values (◆) of the thermodynamic activity of monomer at a series of total concentrations found by the Ω analysis with a solid curve calculated in the following way for each value of a_1. The values of the activities of polymers up to $i = 30$ were calculated using the reported values of k_α and k_β, all values of a_i were divided by the appropriate y_i to give the weight concentration c_i of each species, and these values were summed to give the theoretically predicted abscissa value $\bar{c}$. As can be readily seen from fig. 3, the fit of the theoretical curve to the experimental points is excellent over the entire range of total concentration examined. Indeed, the observed standard deviation between the observed and calculated values of $\bar{c}$ is only 0.017 g/l which is slightly above the experimental precision with which a fringe displacement may be measured on an interferogram (0.01 g/l). Evidently, the head-to-head and tail-to-tail association pattern is consistent with the results obtained at the acid pH.

4.3. Studies at pH 10

The selection of pH 10.0 for an examination of the self-association behaviour of zinc-free insulin in the alkaline range was guided by two considerations. Firstly, pH 10.0 is sufficiently above the pK_a values of all readily ionizable groups in both interaction domains identified in the crystal structure [25,27] to permit an examination of a possible dependence, on pH, of the values of k_α and k_β (if these continue to pertain). Secondly, it has been shown [28] that the base-catalysed cleavage of the inter- and intra-chain disulphide bridges, within the insulin structure, is negligible at pH 10.0 and only becomes a complicating factor at higher pH values around 13. The discussion which follows refers to two sedimentation equilibrium experiments carried out at pH 10 and whose details are given in fig. 4. The uppermost points (■) in fig. 4 are the results of the Ω analysis, which, consistent with the results obtained at lower pH values, yielded overlap of curves relevant to the different experiments conducted to explore the indicated range of total concentration. In relation to corrections made for thermodynamic non-ideality, it suffices to note that the uppermost solid curve in fig. 4 was calculated as previously described. The

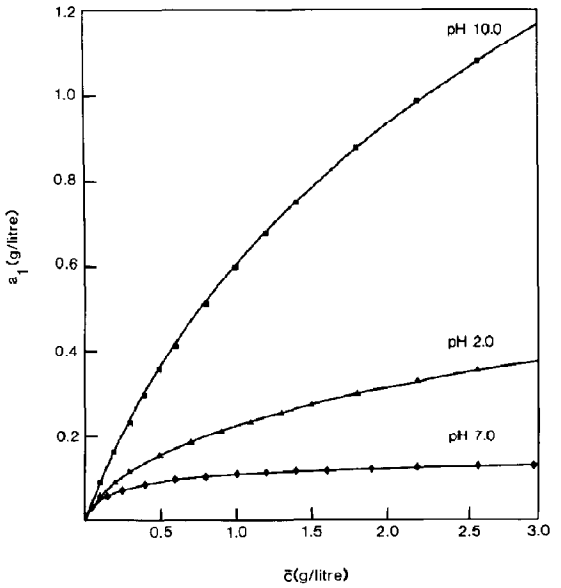

Fig. 4. A composite diagram which shows the dependence on total zinc-free insulin concentration of the thermodynamic activity of insulin monomer relevant to solutions $I = 0.10$, 25° C and at the pH values shown against each curve. The experimental conditions at pH 2 and pH 7 are given in the legends to figs. 1 and 2. The results of two experiments at pH 10 are included: $c_0 = 0.662$ g/l, $\omega = 5450$ rad/s, meniscus depletion; $c_0 = 1.870$ g/l, $\omega = 3145$ rad/s, short column. In each case the solid curves are those calculated on the basis of the refined estimates of k_α and k_β reported in table 2, due allowance having been made for composition-dependent thermodynamic non-ideality effects. The figure is designed to show the appropriateness of the head-to-head and tail-to-tail association pattern for zinc-free insulin over a wide range of pH.

values of k_α and k_β found to be appropriate at pH 10.0 and used to construct the solid curve referred to in fig. 4 were $k_\alpha = 0.23 \times 10^4$ M^{-1} and $k_\beta = 0.04 \times 10^4$ M^{-1}. Evidently the fit of the theoretical curve to the experimental points is excellent.

The important point emerges that all experimental results obtained at pH 2.0, pH 7.0 and pH 10.0, summarized in fig. 4 are fitted, when realistic account has been taken of thermodynamic non-ideality, by a single self-association pattern governed by two association constants k_α and k_β. It is not suggested that the magnitudes of k_α and k_β are invariant with pH and to stress the point values already reported are summarized in table 2. Even before a detailed examination of these values of k_α and k_β is made, it is evident from fig. 4 that all total concentrations examined the weight fraction of monomer increases in the order pH 10.0 > pH 2.0 > pH 7.0. In other words, the overall extent of association is maximal at neutral pH and increases in the order pH 7.0 > pH 2.0 > pH 10.0, a point made graphically in fig. 5 in terms of reduced weight-average molecular weights calculated using eq. 7 with appropriate values of k_α and k_β.

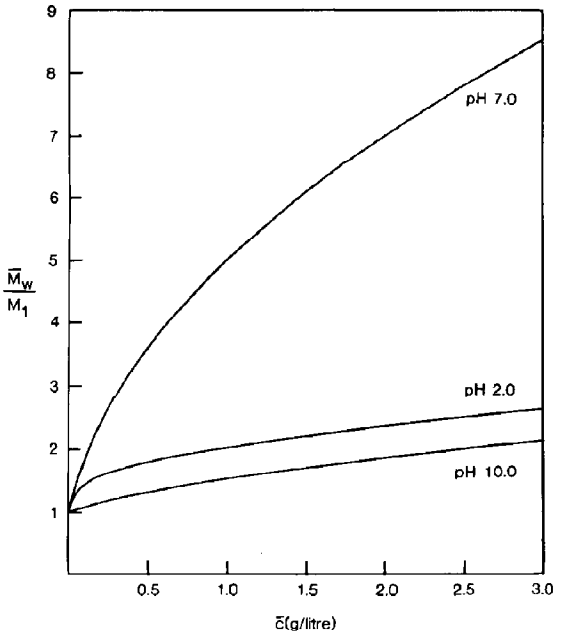

Fig. 5. The overall extent of association of zinc-free insulin at different pH values (as indicated) reflected in plots of the concentration dependence of the reduced weight-average molecular weight. The curves were computed using eq. 7 with the values of k_α and k_β reported in table 2.

Table 2

Summary of the values of k_α and k_β pertinent to the head-to-head and tail-to-tail association pattern of zinc-free insulin found from the analysis of sedimentation equilibrium results

pH	I	T (°C)	k_α (M^{-1}) ($\times 10^{-4}$)	k_β (M^{-1}) ($\times 10^{-4}$)
2.0	0.10	25	3.80	0.03
7.0	0.20	25	5.75 [a]	0.85 [a]
7.0	0.10	25	2.05	1.63
7.0	0.05	25	1.52	1.40 [b]
7.0	0.10	37	3.85	0.39 [b]
10.0	0.10	25	0.23	0.04

[a] Values reported by Nichol et al. [11].

[b] Experimental parameters for all other experiments are reported in the legends to figs. 1–3. Two experiments were performed at pH 7.0, $I = 0.05$, 25 °C and pH 7.0, $I = 0.1$, 37 °C. The relevant parameters were at $I = 0.05$; $c_0 = 0.879$ g/l, $\omega = 5029$ rad/s, meniscus depletion and $c_0 = 1.080$ g/l, $\omega = 2094$ rad/s, short column: at $T = 37$ °C; $c_0 = 1.071$ g/l, $\omega = 4610$ rad/s, meniscus depletion and $c_0 = 1.055$ g/l, $\omega = 2095$ rad/s, short column.

4.4. Studies on the effect of variations of ionic strength and temperature

Table 2 includes values of k_α and k_β reported by Nichol et al. [11] pertaining to sedimentation equilibrium studies performed at pH 7.0, 25 °C and at an ionic strength of 0.20, together with additional results obtained in this work which refer to the same conditions except that the ionic strength was 0.05. The latter results were obtained in an entirely analogous manner to that described previously and were in all respects similar in form to those shown in fig. 4. It is evident from table 2 that, at constant pH and temperature (pH 7.0, 25 °C), an increase of ionic strength results in a small systematic increase in k_α, while the value of k_β remains essentially constant within experimental error, $1.3 \pm 0.4 \times 10^4$ M^{-1}.

The 'physiological' pH of 7.0 was also employed to investigate the association pattern at the

elevated temperature of 37 °C. The results in rows 3 and 5 of table 2 may be compared in this regard since both were obtained at the same ionic strength of 0.10. While little significance can be attached to the numerical values of the standard enthalpy changes calculable from these two sets of results, it may be concluded within experimental precision that an increase of temperature results in an increase in k_α ($\Delta H^\circ > 0$) and to a decrease in k_β ($\Delta H^\circ < 0$). It is relevant to note that for the additional experiments reported in this section the head-to-head and tail-to-tail association continued to provide an excellent description of all results.

5. Discussion

Several points merit comment in relation to the sedimentation equilibrium studies on zinc-free insulin. It is possible to be somewhat critical concerning the nature of the assumptions on which the thermodynamic non-ideality effects are assessed, basically in terms of covolume and charge-charge interaction effects. Both spherical geometry and charge conservation on polymerization have been assumed, the species being viewed as hard impenetrable spheres with Stokes radii taken as the effective radii appropriate to excluded-volume calculations. Nevertheless, it must be stressed that the effects of non-ideality in the present system are not large due to the relatively small size of, and net charges borne by, the protein and because of the restricted range of total concentration (never exceeding 6 g/l) which was examined. The consideration of non-ideality effects is both realistic and important with certain systems, but it is evidently a second-order effect in the present system. The matter is put into perspective by observing that the activity coefficient of haemoglobin at the erythrocyte concentration of 320 g/l [29] has been assessed as 62.8, whereas the largest value found in the present study is 3, this value referring to a decamer of insulin in relatively low amount present at acid pH where charge-charge interactions were maximal in this study. Accordingly, there is less concern with the approximations used in the statistical-mechanical calculation of non-ideality effects than with the requirement that some realistic account be taken of them in refining values of the equilibrium constants governing the association of zinc-free insulin. It should be noted that the determination of the thermodynamic activity of monomer is independent of any assumption concerning the nature of the self-association pattern and provides the basis for the iterative method used to calculate the composition dependence of the activity coefficients in relation to an assumed model of association.

The major point which emerges from the sedimentation equilibrium studies is that a single association pattern, termed head-to-head and tail-to-tail, suffices to describe all results found over a wide pH range and (at pH 7.0) at different ionic strengths and temperatures. It is not suggested that each of these results viewed separately might not be fitted by an alternative model of association, indeed this has been done by various groups of workers and has led to a wide spectrum of patterns, each claimed to be operative in a particular environment. It is, however, suggested that the weight of present evidence, including that available from X-ray crystallographic studies, strongly supports the view that the insulin monomer is bivalent and that it associates in the absence of Zn^{2+} proceeding in an indefinite fashion by homogeneous bivalent interaction at two independent sites. The postulate does not invoke exclusion of particular polymeric species, such as odd-numbered polymers [4], nor does it require that emphasis be given to a particular species such as the zinc-free hexamer [9]. Indeed, it suggests an undeniably complicated detailed composition of each solution in which two types of even-numbered polymers are present (with identical but different interaction domains at each end) and all odd-numbered polymers (with different interaction domains at each end) coexist in equilibrium with the monomer. The pertinent point is, despite this complexity, that the detailed composition may be described in quite simple terms by eq. 3, which involves only two thermodynamic constants, k_α and k_β. The fitting procedure used to obtain these quantities reported in table 2 is quite sensitive and in this respect the precision given is warranted; but, at the same time, it is readily acknowledged

that the experimental points derived from the Ω analysis, which have been fitted, are themselves subjected to experimental error and thus it may be assessed that each reported value has an uncertainty of approx. 15%. The ability to fit all experimental results within experimental error to the two parameters k_α and k_β highlights the likely appropriateness of the head-to-head and tail-to-tail association pattern in describing the solution behaviour of zinc-free insulin. This finding may have general implication in relation to other self-associating protein systems, such as lysozyme and chymotrypsinogen A, where different association patterns have been invoked to explain results obtained in experimental environments [21,30].

A primary reason for formulating the present association pattern for insulin in solution was the indentification of two different sets of interactions between monomers in the crystallographically determined zinc hexamer structure. These are defined as interactions about two 2-fold axes, OP and OQ, respectively [27]. In a previous limited examination of the likely validity of the model utilizing experimental results obtained at pH 7.0, Nichol et al. [11] noted that symmetry of eq. 3 obviated unequivocal identification of k_α and k_β with either of these interaction domains. However, those workers concluded, after consideration of the relative magnitudes of k_α and k_β, that it was likely that they referred to the interactions about OP and OQ, respectively. The present work allows that conclusion to be tested more stringently. In addition, examination of the variation of k_α and k_β with pH, ionic strength and temperature might provide further support for the postulated association pattern in that trends should be at least consistent with the chemistry of constituent residues in the reaction domains, once defined. At the outset it is observed that the standard free energy changes associated with k_α and k_β are the sum of several interaction components, not least the free energy of repulsion between species of like charge. In this connection it is noted that the overall extent of association is greatest at pH 7.0 where the net charge repulsion is at a minimum (fig. 5). It follows that a detailed interpretation of standard free energy changes is somewhat hazardous; but, with this reservation in mind, the following points are made. The value of k_α increases with increased temperature ($\Delta H^\circ > 0$) and with increasing ionic strength, both characteristics of interactions which are predominantly hydrophobic in nature [31]. This would suggest that the α-α interaction may be identified with reaction between groups in the OP domain. These groups are predominantly hydrophobic and form a region of secondary structure which is highly conserved between the various crystal structures obtained with porcine insulin in the presence and absence of Zn^{2+} [27,32,33]. In these terms k_α (which in all experimental environments is greater than k_β) governs the equilibria forming the α-α (OP-OP) dimer and the addition of monomers also at the OP interface, to a free OP interface in the formation of higher polymers. The relatively small dependence of k_α on pH (compared with k_β), at fixed ionic strength and temperature, is consistent with this assignment and, moreover, the variation itself finds rational explanation when it is appreciated that the only ionizable groups in the OP domain of 2-zinc insulin are glutamic acid B13 (pK_a 4.7) and tyrosines B24 and B26 (pK_a 9.6) [25,27]. From table 2 it is clear that as the pH is increased from pH 2.0 to pH 10.0 the value of k_α decreases in accord with an increasing state of ionization of the three groups and the inherent increase in like charge repulsion opposing the hydrophobic attractions. The apparent corollary is that the formation of the β-β dimers and higher polymers involving like interfaces, governed by k_β, is associated with interactions of groups in the OQ domain which notably feature the ionizable groups tyrosine A14, phenylalanine B1 and glutamic acid A17. It is tempting to suggest that the negative enthalpy change associated with k_β, the maximal value of k_β at pH 7.0, and the slight decrease in k_β with increasing ionic strength at pH 7.0 are all consistent with interaction between hydrophilic groups especially glutamic acid A17 and the amino-terminal phenylalanine B1 which bear opposite net charges at pH 7.0. However, a cautionary note is sounded in that such detailed interpretation in terms of the overall 'free-energy balance sheet' is not strictly warranted, especially as it is by no means certain that the groups shown in the OQ domain are necessarily those involved

in crystal formation or associated states of zinc-free insulin in free solution. In this connection it is noted that zinc-free porcine insulin crystallizes in the form of linear chains along a screw axis [33]. Despite a warranted reluctance to provide a detailed interpretation of all variation shown in table 2, it is fair to say that all observations at least find a rational explanation in terms of the head-to-head and tail-to-tail association pattern, that almost certainly the α-α interactions may be identified with OP-OP interactions and that while the details of the β-β interactions in solution remain to be elucidated, they most certainly exist.

In summary, the questions posed in section 1 be answered as follows. It is indeed possible to describe the solution behaviour of zinc-free insulin in terms of a single association pattern over the range from pH 2 to pH 10. In general, the solution will contain an equilibrium mixture of all polymeric forms of insulin of composition depending on the total concentration and the values of k_α and k_β appropriate to the environment considered. The hexamer is merely one of those species and has no particular significance. Variations of the site-binding constants may be explained satisfactorily in terms of the known properties of amino acid residues in the postulated reaction domains, thus supporting the proposed model. It is possible to see how variations in the relative magnitudes of k_α and k_β, by favouring the formation of some species and almost eliminating others, can explain the success of other models in fitting experimental results under more limited sets of conditions.

Finally, in order to place these findings into a biological context it should be remembered that in humans the normal resting concentration of insulin in serum is approx. 0.7 ng/ml, rising to 3.0 ng/ml after the infusion of a glucose load [34]. Maximal cell stimulation by insulin occurs by 10 ng/ml [35]. Under the same conditions used to calculate the weight fractions of the species plotted in fig. 2, a solution containing 10 ng/ml of insulin would comprise 99.987% monomer by weight. In this respect the properties of the present model for the self-association of insulin do not alter the conclusion of previous workers [4,36] that the self-association is not relevant to insulin's actions in vivo. However, it should be remembered that the association constants have been derived for solutions of pure insulin in aqueous buffers and that little or no attention has been given to naturally occurring constraints which may perturb the operative equilibria under physiological conditions.

References

1 J.H. Bradbury, V. Ramesh and G.G. Dodson, J. Mol. Biol. 150 (1981) 609.
2 J. Goldman and F.H. Carpenter, Biochemistry 13 (1974) 4566.
3 E. Fredericq, Arch. Biochem. Biophys. 65 (1956) 218.
4 P.D. Jeffrey, B.K. Milthorpe and L.W. Nichol, Biochemistry 15 (1976) 4660.
5 R.F. Steiner, Arch. Biochem. Biophys. 39 (1952) 333.
6 P.D. Jeffrey and J.H. Coates, Biochemistry 5 (1966) 489.
7 L.A. Holladay, M. Ascoli and D. Puett, Biochim. Biophys. Acta 494 (1977) 245.
8 G.M. Wu, Dissertation Abstracts 35 (75) (1974) 7816.
9 A.H. Pekar and B.H. Frank, Biochemistry 11 (1972) 4013.
10 A. Wollmer, W. Strassburger, E. Hoenjet, U. Glattner, J. Fleischauer, D.A. Mercola, R.A.G. de Graaf, E.J. Dodson, G.G. Dodson, D.G. Smith, D. Brandenburg and W. Danho, in: Insulin: chemistry, structure and function of insulin and related hormones, eds. D. Brandenburg and A. Wollmer (Walter de Gruyter, Berlin, 1980) p. 27.
11 L.W. Nichol, M.J. Sculley, P.D. Jeffrey and D.J. Winzor, J. Theor. Biol. 109 (1984) 285.
12 R.E. Chance, M.A. Root and J.A. Galloway, Acta Endocrinol. Suppl. 205 (1976) 185.
13 B.H. Frank and A.J. Veros, Biochem. Biophys. Res. Commun. 32 (1968) 155.
14 B.K. Milthorpe, Ph.D. Thesis, Australian National University (1977).
15 E.G. Richards, D.C. Teller and H.K. Schachman, Biochemistry 7 (1968) 1054.
16 C.H. Chervenka, Anal. Biochem. 34 (1970) 24.
17 E.F. Casassa and H. Eisenberg, Adv. Protein Chem. 19 (1964) 287.
18 B.K. Milthorpe, P.D. Jeffrey and L.W. Nichol. Biophys. Chem. 3 (1975) 169.
19 H.B. Dwight, Tables of intergrals and other mathematical data, 4th edn. (Macmillan, New York, 1961).
20 L.-H. Tang, D.R. Powell, B.M. Escott and E.T. Adams Jr Biophys. Chem. 7 (1977) 121.
21 P.R. Wills, L.W. Nichol and R.J. Siezen, Biophys. Chem. 11 (1980) 71.
22 L.W. Nichol and D.J. Winzor, J. Phys. Chem. 80 (1976) 1980.
23 A.P. Ryle, F. Sanger, L.F. Smith and R. Kitai, Biochem. J. 60 (1955) 541.
24 P.D. Jeffrey, L.W. Nichol. D.R. Turner and D.J. Winzor, J. Phys. Chem. 81 (1977) 776.

25 C. Tandford and J. Epstein, J. Am. Chem. Soc. 76 (1954) 2163.
26 P.D. Jeffrey, in: Protein-protein interactions, eds. C. Frieden and L.W. Nichol (Wiley, New York, 1981) p. 213.
27 T.L. Blundell, G.G. Dodson, D.C. Hodgkin and D.A. Mercola, Adv. Protein Chem. 26 (1972) 279.
28 E. Helmerhorst and G.B. Stokes, Biochemistry 22 (1983) 69.
29 P.D. Ross and A.P. Minton, J. Mol. Biol. 112 (1977) 437.
30 M.S. Tung and R.F. Steiner, Eur. J. Biochem. 44 (1974) 49.
31 W. Kauzmann, Adv. Protein Chem. 14 (1959) 1.
32 G.D. Smith, D.C. Swenson, E.J. Dodson, G.G. Dodson and C.D. Reynolds, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7093.
33 E.J. Dodson, G.G. Dodson, A. Lewitova and M. Sabesan, J. Mol. Biol. 125 (1978) 387.
34 S.S. Fajans and J.C. Floyd Jr, in: Handbook of physiology, ed. S.R. Geiger, (American Physiological Society, Washington, DC, 1972) section 7, vol. 1, p. 473.
35 J.W. Marsh, J. Westley and D.F. Steiner, J. Biol. Chem. 259 (1984) 6641.
36 P. de Meyts, A.R. Bianco and J. Roth, J. Biol. Chem. 251 (1976) 1877.