Insulin Self-Association. Spectrum Changes and Thermodynamics†

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ABSTRACT: Concentration difference spectra have been measured for insulin at 0.1 ionic strength, pH 2 and 15 to 44°; at 0.1 ionic strength, pH 3.5 and 26°; and at 0.01 ionic strength, pH 2 or 3.5 and 26°. The spectrum change reflects association of monomer to dimer and there is no significant contribution from formation of higher polymers. The concentration dependence of the difference spectrum determines the equilibrium constant for the monomer–dimer reaction under the various conditions cited. The thermodynamic parameters for insulin dimerization at pH 2, 25°, and 0.1 ionic strength are $\Delta F_u = -8.7$ kcal, $\Delta H° = -17.2$ kcal, and $\Delta S_u = -29$ cal/(degree mol). A change in pH from 2 to 3.5 weakens dimerization by a factor of 1.3 and a change in ionic strength from 0.1 to 0.01 weakens dimerization by a factor of 4. Spectrum changes associated with change in pH or ionic strength are similar although not identical for the monomer and dimer. Changes in the tyrosine spectrum of comparable magnitude are brought about through folding from the random coil to the insulin monomer, through dimerization of the monomer, and through increase in pH above 2. Changes in the far-ultraviolet circular dichroic spectrum are associated with dimerization and reflect in part the formation of intersubunit β structure. Addition of zinc ion to zinc-free insulin does not affect spectrum or association behavior at pH 3.5 and 0.1 ionic strength.

The self-association of insulin is one of the first protein–protein interactions to have been examined (Sjögren and Svedberg, 1931). It subsequently received substantial attention (reviewed by Reithel, 1963), the most recent studies being the sedimentation equilibrium measurements of Jeffrey and Coates (1966a,b) and of Pekar and Frank (1972). The insulin monomer (5733 daltons) associates strongly to a dimer, which at higher concentrations polymerizes to give predominantly tetramers and hexamers. At pH near 2, low ionic strength, and temperature near 25° the dimer even at concentrations of 10 mg/ml is the principal associated species. Increasing the ionic strength or raising the pH to 4, the lower limit of the region of insulin insolubility, favors formation of tetramer and hexamer. Insulin, a small protein with complex association behavior, is well suited for detailed definition of interactions important in subunit structures. The high-resolution crystallographic analysis of insulin (Blundell *et al.*, 1971) makes this protein a particularly attractive system for study.

Fischer and Cross (1965) proposed that difference spectrum measurements (concentration difference spectra) could be of general importance in studies of protein association. These have been used in work with hemoglobin (Mizukami and Lumry, 1967), glucagon (Blanchard and King, 1966; Swann and Hammes, 1969), and insulin (Rupley *et al.*, 1967). The absorbance of a solution of protein at one concentration is measured against a reference solution of different concentration, using cells of pathlengths chosen so that the same total mass of protein is in the sample and reference beams of the spectrophotometer. Because of the sensitivity of absorbance measurements, data of this kind can be obtained at a high dilution of protein. In this connection association data for the rather stable insulin dimer have not been obtained previously under conditions (below 0.1 mg/ml) where the major fraction of the protein is monomer.

Rupley *et al.* (1967) have reported that dimerization of insulin alters its spectrum, but that higher polymer formation has no such effect. The experiments to be described characterize insulin dimerization in dilute solution, through correlating changes in absorbance spectrum with total protein concentration, pH, and ionic strength. The data are consistent with an equilibrium constant for pH 2 and 0.1 ionic strength that is somewhat greater than that calculated by Jeffrey and Coates (1966a) and show that both the spectrum change and dimerization equilibrium are affected by change in pH or ionic strength.

Experimental Section

Protein solutions were prepared from crystalline bovine zinc insulin (Eli Lilly). Protein concentration was determined spectrophotometrically using ϵ_M^{280} 5220 for zinc insulin at 0.1 ionic strength, pH 2, and *ca.* 1 mg/ml of insulin (Praissman and Rupley, 1968). The pH was measured with a Radiometer TTTlc instrument.

Buffer was not used in protein solutions at pH 2, and ionic strength was adjusted with NaCl. At pH 3.5 the buffers were 0.05 M NaCl–0.05 M sodium acetate for ionic strength 0.1, and 0.01 M sodium acetate for ionic strength 0.01. Water was deionized using a Continental system.

Spectrophotometry. Studies on the relationships between changes in pH or ionic strength and dimerization were done at 26 ± 1° with a Cary Model 14R recording spectrophotometer equipped with a 0–0.1 absorbance slide-wire. Concentration difference spectra at 15–44° were measured with a Cary Model 15 recording spectrophotometer equipped with water-jacketed V-block cell holders (thermocouple calibration). Cells were from Pyrocell or Precision and were calibrated (±0.002 cm), using a Unitron measuring microscope.

Spectropolarimetry. Circular dichroism spectra were re-

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corded at 27° on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment. Slits were programmed for a 15-Å bandwidth at each wavelength; 1.0- and 0.01-cm cylindrical cells were used. Buffer base lines were determined after each measurement. Each sample and base-line spectrum was scanned twice.

Concentration Difference Spectra. Two cells of different pathlengths (l_1 and l_2) were filled with solutions of concentrations (c_1 and c_2) such that the products c_1l_1 and c_2l_2 were equal (*e.g.*, a 10-cm cell filled with 0.02 mg/ml of protein solution was measured against a 0.1-cm cell with 2 mg/ml of protein). Five–seven different pathlengths were used in a series of constant *cl*. One cell, usually 0.1 or 1.0 cm, was chosen as the reference for a series. For each series, two sets of difference spectra were recorded, one with the reference solution in the reference compartment, the other with cells interchanged. An instrument blank was measured with identical 1-cm cells filled with protein solution, and base lines with buffer in all cells were measured after each series of experiments. Peak heights were corrected for the corresponding protein blank and buffer base-line absorbances. Values of the absorbance differences (ΔA) were obtained as an average of the two spectra for a given pair of cells.

A single series of constant *cl* gave a set of values of ΔA that were converted into differences in extinction coefficient ($\Delta\epsilon^{obsd} = \Delta A/cl$). A plot of $\Delta\epsilon$ *vs.* concentration for a single *cl* series corresponds to a segment of the total curve that represents $\Delta\epsilon$ as a function of protein concentration. The whole curve was obtained by combining several overlapping series of different *cl* (see below).

Difference spectra were also used to establish the effects of pH and ionic strength. These were measured in the usual way, with sample and reference cells of identical pathlength. Measurements at high or low protein concentration were with 0.1- or 10-cm cells. In these cases, to check on the matching of cell pathlength, solutions were sometimes switched between cells of a pair, and the spectrum was rescanned. The differences between the two spectra were negligible.

Association constants were calculated from the variation of $\Delta\epsilon^{obsd}$ with total protein concentration. Assuming a monomer–dimer equilibrium, the equilibrium constant for dimer formation, K_{12}, defined for unit molarity standard state can be expressed in weight concentration units as

$$K_{12} = \frac{[\text{dimer}]}{[\text{monomer}]^2} = \frac{1/2(C_0 - C_1)}{(C_1)^2}M_1 \qquad (1)$$

where C_1 and C_0 are the concentrations (milligrams/milliliter) of monomer and total protein and M_1 is the molecular weight of monomer. Assuming that $\Delta\epsilon$ develops because of dimer formation, the concentration dependence of $\Delta\epsilon$ with respect to an infinitely dilute reference solution can be expressed as

$$\Delta\epsilon = \Delta\epsilon^\circ[(C_0 - C_1)/C_0] \qquad (2)$$

where, from eq 1

$$C_1 = (M_1 + \sqrt{M_1^2 + 8K_{12}M_1C_0})/4K_{12} \qquad (3)$$

and $\Delta\epsilon^\circ$ is the total change in extinction coefficient for conversion of monomer entirely to dimer. Because the protein in the reference cell is not infinitely dilute, the calculated change in extinction coefficient ($\Delta\epsilon^{calcd}$) that corresponds to the observed change ($\Delta\epsilon^{obsd}$) is

$$\Delta\epsilon^{calcd} = \Delta\epsilon^s - \Delta\epsilon^r$$

$$\Delta\epsilon^{calcd} = \Delta\epsilon^\circ[C_1^r/C_0^r - C_1^s/C_0^s] \qquad (4)$$

where $\Delta\epsilon^s$ and $\Delta\epsilon^r$ are determined as in eq 2. The values of K_{12} and $\Delta\epsilon^\circ$ which fit the data were evaluated by using eq 3 and 4 and an iterative nonlinear least-squares analysis (a modified form of the program developed by Moore and Zeigler, 1960). In the plots of $\Delta\epsilon$ *vs.* protein concentration given below, the curves are calculated according to eq 2 and data points are adjusted to infinitely dilute reference solution.

Association of insulin to polymers larger than dimer was taken into account in some attempts to fit the data, using an appropriate modification of eq 3. No significant improvement over the assumption of a monomer–dimer equilibrium in the fit of $\Delta\epsilon^{calcd}$ to $\Delta\epsilon^{obsd}$ was obtained. This accords with expectation from the weakness of the association to higher oligomers (Jeffrey and Coates, 1966a). At 2 mg of protein/ml, pH 2, 25°, and ionic strength 0.1, insulin is *ca.* 80% dimer and there is less than 10% tetramer and a negligible amount of hexamer.

Data measured over a range of temperature were treated in the following manner. Temperature dependence was incorporated into eq 3 as

$$K_{12}(T) = K_{12}(298.2°)e^{\frac{\Delta H^\circ}{RT(298.2^\circ)}(T - 298.2^\circ)} \qquad (5)$$

The variation with temperature of $\Delta\epsilon^\circ$ for dimerization was approximated by

$$\Delta\epsilon^\circ(T) = \Delta\epsilon(298.2°) + A(T - 298.2°) \qquad (6)$$

The constant A ($d\epsilon(\text{dimer})/dT - d\epsilon(\text{monomer})/dT$) was determined by measuring temperature difference spectra for monomer and dimer. At experimentally accessible concentrations of protein some monomer is present in solutions predominantly dimer (20 mg/ml) and some dimer in solutions predominantly monomer (0.05 mg/ml). Thus the extinction difference in a temperature difference spectrum measurement is a function of the association parameters ($\Delta\epsilon(298.2°)$, K_{12}-(298.2°), and ΔH°) as well as the temperature difference. The values of $d\epsilon(\text{dimer})/dT$ and $d\epsilon(\text{monomer})/dT$ were determined by fitting temperature difference data with a function that included estimated values for the association parameters. The best value of A so determined was then used to refine the values of the association parameters, through a least-squares fit of eq 3–6 to the concentration difference spectrum data. This cycle was repeated until the values of all parameters showed no significant change.

Errors in $\Delta\epsilon$ came from the following sources: uncertainty in *cl*, instrument noise, and light scattering by the protein. The estimated error in the product *cl* is 0.5% or less, which corresponds to 10 or less in $\Delta\epsilon$ at 286 nm. Instrument noise was significant at low protein concentration, *i.e.*, for $cl = 0.2$ this contribution to the error in $\Delta\epsilon$ is about 30. Systematic errors in instrument operation were not detected; an increase in the slit width and reversal of the cells did not change the spectrum. Light scattering by the protein was apparently not significant under the conditions of these measurements, a conclusion from the flat base line above 300 nm. Leach and

TABLE I: Difference Spectrum Data for Perturbation of Insulin and Dimerization Equilibrium Constants.

T (°C)	pH	μ	M or D	λ_{max} (nm)[a]	$\Delta\epsilon^\circ_{286}$[b]	$\Delta\epsilon^\circ_{260}$	$K_{12} \times 10^{-4}$ [c] (M^{-1})	No. of Data Points	Concn Range (mg/ml)
				A. Dimerization					
15	2.0	0.1		286.0	490		10.9 (0.77)	5	0.05–2.7
25	2.0	0.1		286.0	480	25	4.0 (0.18)	51	0.01–9.4
30	2.0	0.1		286.0	475		1.8 (0.37)	5	0.34–17.2
35	2.0	0.1		285.5	470		1.9 (0.17)	19	0.05–9.7
44	2.0	0.1		285.5	460		0.61 (0.07)	27	0.06–16.4
26	3.5	0.1		286.0	500	15	3.2 (0.52)	24	0.02–18.9
26	2.0	0.01		285.5	535	55	1.1 (0.19)	25	0.04–18.4
26	3.5	0.01		285.5	405	15	0.75 (0.26)	18	0.05–19.3
				B. Change of Ionic Strength, 0.1 → 0.01					
26	2.0		M	285.0	170	20			
26	2.0		D	287.0	115	5			
				C. Change of pH, 2.0 → 3.5					
26		0.1	M	286.0	190	10			
26		0.1	D	286.5	210[d]	5			
26		0.01	M	286.0	295	30			
26		0.01	D	286.5	210	10			
				D. Change of Temperature, 25 → 44° [e]					
	2.0	0.1	M	288.5	130	14			
	2.0	0.1	D	288.0	110	17			
				E. Folding, Random Coil to Dimer[f]					
25	2.0	0.1	D	287.0	710	70			

[a] The average of the peak wavelength determined for about ten spectra. Values were rounded off to the nearest 0.5 nm. [b] A standard error of 10–25 extinction units in $\Delta\epsilon^\circ_{286}$ was obtained using the fitting procedure described in the text. $\Delta\epsilon^\circ$ first was allowed to vary in fitting the full set of data at pH 2, $\mu = 0.1$, and various temperatures. Then $\Delta\epsilon^\circ$ was fixed for the separate fits of the data at each temperature to obtain the estimates of K_{12}. [c] Association constants calculated from concentration difference spectra assuming a monomer–dimer (M–D) equilibrium. The values in parentheses are standard deviations. [d] The pK of 3.5 measured for this spectrum change (Laskowski *et al.*, 1960) suggests that a total $\Delta\epsilon$ of ~400 is developed by protonation of the group(s) in question. [e] Absorbance changes corrected for contribution of association or dissociation. [f] Determined for 0.92 mg/ml of insulin, at which concentration the protein is 79% associated.

Scheraga (1960) found important light scattering contributions at pH values above 2. The low scattering in the present experiments probably owes to the generally low values of *cl*. Thus, the estimated total error in $\Delta\epsilon$ is *ca.* 20, except at very low protein concentration, where it can be about twice that. Because errors in pH and ionic strength difference spectra derive essentially only from *cl*, the error should be about 10 in $\Delta\epsilon$.

Results

Concentration Difference Spectra. Concentration difference spectra were measured for zinc insulin at pH 2 and 3.5 and ionic strength 0.01 and 0.1. At pH 2 and ionic strength 0.1, measurements were made at 15, 25, 30, 35, and 44°. Figure 1 shows typical traces of the Cary records. The characteristic 286- and 279-nm extremes correspond to tyrosine perturbation (Wetlaufer, 1962). The structure at lower wavelengths (maxima *ca.* 249, 254, 260, 266, and 270 nm) is associated with perturbation of phenylalanine side chains (Wetlaufer, 1962). Table I lists the values of λ_{max}, K_{12}, and $\Delta\epsilon$ found for the self-association reaction and the other perturbations studied in this work. The parameters change with solution conditions, *i.e.* temperature, pH, and ionic strength. $\Delta\epsilon_{260}$, which presumably reflects changes about phenylalanine residues, was measured as the height of the peak at 260 nm above a line drawn between the adjacent two troughs in the spectrum. The tyrosine contribution present as a background at 260 nm thus should be largely eliminated.

Absorbance Changes as a Function of Zinc Insulin Concentration. Figure 2 shows the variation with concentration of $\Delta\epsilon_{286}$ for zinc insulin at pH 2, $\mu = 0.1$, and 25, 35, and 44°. Table IA gives values of K_{12} and $\Delta\epsilon^\circ$ fit to the data for these and other conditions of pH, ionic strength, and temperature. The temperature range over which association measurements could be made was limited by fibril formation at temperatures above 45° (Waugh, 1954) and by instrument sensitivity at low temperatures, where stronger association requires high dilution for dissociation of dimer.

Effect of Temperature on Absorbance. Figure 3 shows that the extinction coefficient changes with temperature less for dimer than for monomer. Data points (open symbols) are values from temperature difference spectra measured at high (20 mg/ml) and low (0.05 mg/ml) insulin concentration, conditions under which the protein is predominantly dimer and monomer, respectively. However, the fraction of protein present as dimer also changes with temperature, and this can make an important contribution to the temperature difference spectrum, particularly at 0.05 mg/ml and temperatures below 40°. The filled symbols of Figure 3 are calculated from the data with correction for association or dissociation (see Experimental Section).

The corrected data are adequately described by functions linear in temperature, with slopes 9.1 for dimer and 10.7 for

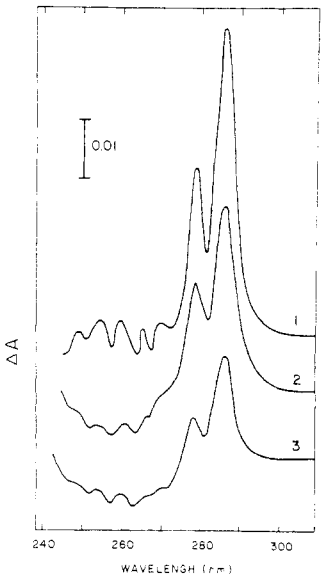

FIGURE 1: Concentration difference spectra of zinc insulin. Curve 1: pH 2, μ = 0.01, 1.84 mg/ml in 1.0-cm cell *vs.* (reference) 0.184 mg/ml in 10-cm cell. Curve 2: pH 3.5, μ = 0.1, 18.9 mg/ml in 0.1-cm cell *vs.* 1.89 mg/ml in 1-cm cell. Curve 3: pH 2, μ = 0.1, 0.87 mg/ml in 1-cm cell *vs.* 0.087 mg/ml in 10-cm cell. These spectra are traced directly from the Cary record without buffer and baseline corrections.

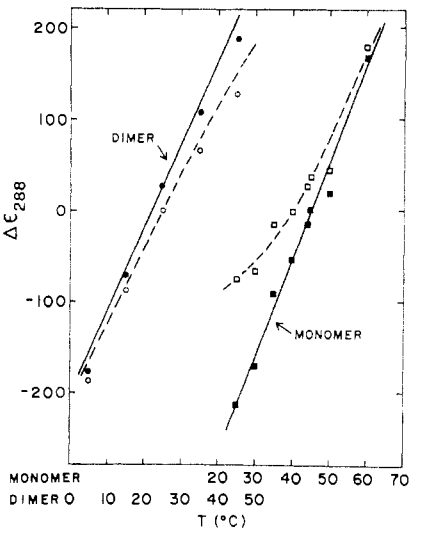

FIGURE 3: Change in extinction coefficient of zinc insulin with temperature at pH 2, μ = 0.1, and 0.05 mg/ml of monomer and 20 mg/ml of dimer: (- - -) calculated for measured concentrations using values estimated for association and absorbance parameters and their temperature dependence; (—) calculated for solutions of pure monomer or dimer; open symbols, experimental data (reference temperature 25° for dimer and 40° for monomer); closed symbols, data corrected for contribution to absorbance difference of changes in association, *i.e.*, for contribution of dimer to monomer solutions and *vice versa*.

monomer. The greater temperature dependence of monomer absorbance is in accord with greater exposure of tyrosyl residues to solvent in the monomer (Bello, 1969). The absorbance change for conversion of monomer to dimer will vary with temperature to the extent that the extinction coefficients for monomer and dimer change differently with temperature. The difference between the slopes is −1.6 molar extinction units/degree at 288 nm. The difference is

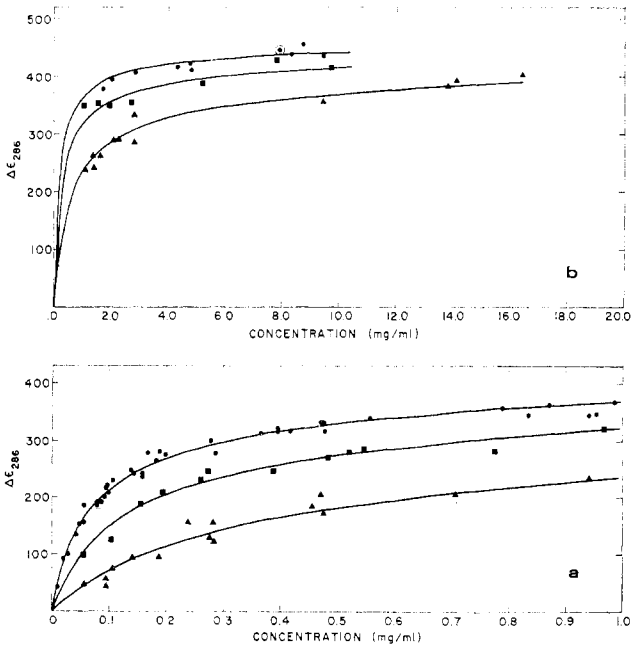

FIGURE 2: Change in extinction coefficient with zinc insulin concentration at pH 2, μ = 0.1, and 25 (●), 35 (■), and 44° (▲): (a) expanded 0–1.0-mg/ml concentration range; (b) 0–20-mg/ml concentration range. The points in b do not include the points in a (below 1 mg/ml). The curves were calculated with the values of K_{12} and $\Delta\epsilon^\circ$ in Table IA.

−1.0 at 286 nm where the concentration difference spectrum data were taken. This is the value of the constant A in eq 6.

Enthalpy of Association. The least-squares analysis of data for all temperatures (107 data points), using eq 3–6 with A = −1.0, gave $\Delta\epsilon(298.2°)$ = 480, $K_{12}(298.2°)$ = 4.04 × 10^4 M^{-1}, and $\Delta H°_{12}$ = −17.2 kcal/mol of dimer. The standard deviation of the fit is 12.3 extinction units, in agreement with the estimated error of the measurements (10–20 extinction units). The essential correctness of this analysis is supported by the values of the association parameters fit to the data separately for each temperature. The temperature dependence of the separately determined values of K_{12} (Figure 4) gives $\Delta H°$ = −17.9 kcal, which is not significantly different from the value −17.2 kcal from the simultaneous fit of all data.

Effect of Ionic Strength on Absorbance. Curve 3 of Figure 5 gives the difference spectrum produced by increase in ionic strength from 0.01 to 0.1 at pH 2 and 1 mg/ml of zinc insulin. Experiments at protein concentrations ranging from 0.1 to 19 mg/ml determined $\Delta\epsilon$ for change from 0.01 to 0.1 ionic strength as 170 for the monomer and 115 for the dimer (Table IB).

Effect of pH on Absorbance. Curves 1 and 2 of Figure 5 show typical difference spectra produced by change in pH from 2 to 3.5 for zinc insulin. The corresponding changes in extinction coefficient are given in Table IC. The effect of change in pH is greater for the zinc insulin monomer at μ = 0.01 than for the protein under other conditions.

Circular Dichroism. The circular dichroic (CD) spectra of zinc insulin at 1.0 and 0.01 mg/ml in 0.01 M $NaClO_4$ and at pH 2.0 are shown in Figure 6. Dilution of a 1.0-mg/ml solution by 100-fold in 0.01 M $NaClO_4$ results in considerable conversion of insulin dimer to monomer (60 to 4% dimer). The CD spectrum of the 0.01-mg/ml solution has a low-wavelength maximum blue-shifted 2.5 nm to 192.5 nm. The intensity of this band is decreased and there is a corresponding increase in intensity of the negative band at 208 nm. The negative band at 222 nm shows a small intensity decrease on

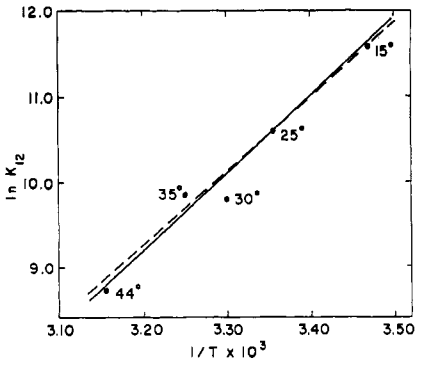

FIGURE 4: Van't Hoff plot of the equilibrium constants listed in Table IA: (—) determined using a weighted least-squares analysis of the five points ($\Delta H^\circ = -17.9$ kcal); (- - -) calculated for $\Delta H^\circ = -17.2$ kcal.

dilution. The difference spectrum (Figure 6) obtained by subtracting the 0.01-mg/ml from the 1.0-mg/ml spectrum has a small negative band at 225 nm and a positive band with a maximum at 195–200 nm. Measurements in 0.05 M $NaClO_4$ at pH 2.0 gave results closely similar to 0.01 M $NaClO_4$.

Comparison of 1.0- and 0.01-mg/ml salt-free solutions adjusted to pH 2.0 with $HClO_4$ showed (Figure 7) similar but substantially larger differences than found for 0.01 M $NaClO_4$. The change with dilution in the amount of dimer is expected to be equal to or less in salt-free solution than in 0.01 M $NaClO_4$. In salt-free solution at 1.0 mg/ml the positive maximum is at 196 nm in agreement with Ettinger and Timasheff (1971a). This maximum shifts 4 nm to 192 nm on dilution to 0.01 mg/ml. The difference spectrum is similar in shape in this case to those for the solutions containing salt, but the magnitude around 198 nm is approximately doubled.

Spectrum Perturbation Associated with Folding from Random Coil to Insulin Monomer. The spectrum change associated with folding of insulin was determined by comparing the spectrum of reduced insulin in 8 M urea with that of the native protein. Because high concentrations of urea perturbed chromophores, spectra were measured at various urea concentrations for extrapolation to determine the properties of the random coil molecule in the experimentally unobtainable state of dilute salt solution.

Insulin (4.6 mg/ml) was allowed to react for 1.5 hr at room temperature in a 0.1 M NaCl–8 M urea–0.8 M mercaptoethanol solution of pH 8. The protein was diluted fivefold with 0.1 M sodium chloride containing appropriate concentrations of urea and acid to give 3.3–9.7 M urea and a final pH of 2. The difference spectrum was determined with a reference solution of native protein in 0.1 M sodium chloride at pH 2. The extinction difference depended linearly on urea concentration (largest deviation: 15 extinction units). Extrapolation to zero concentration of urea and mercaptoethanol gave the change in absorbance for folding of insulin at 0.92 mg/ml as 710 extinction units at 286 nm and 90 extinction units at 260 nm. Insulin is 79% associated to the dimer under the conditions used in this measurement. Correction for association gives the spectrum change for folding to the insulin monomer as 310 extinction units at 286 nm and 70 extinction units at 260 nm.

Effect of Zinc Ion on the Absorbance and Association of Zinc-Free Insulin. The effect of zinc ion on the spectrum of zinc-free insulin was examined at pH 3.5 and $\mu = 0.1$. Addition of a *ca.* fivefold molar excess of zinc ion (10^{-4} M) at 0.098 mg/ml of protein (1.7×10^{-5} M) gave no difference spectrum. A small effect but not one characteristic of tyrosyl perturbation was observed at 100× molar excess (2×10^{-3} M). No significant difference was produced by 2×10^{-3} M zinc at 10 mg/ml of protein (1.7×10^{-3} M). Praissman and Rupley (1968) reported no effect of zinc ion on the spectrum at pH 2. The self-association of zinc-free insulin, examined at pH 3.5 and $\mu = 0.1$ (using two overlapping concentration series, instead of the three or more generally measured), did not differ from that of zinc insulin under the same conditions.

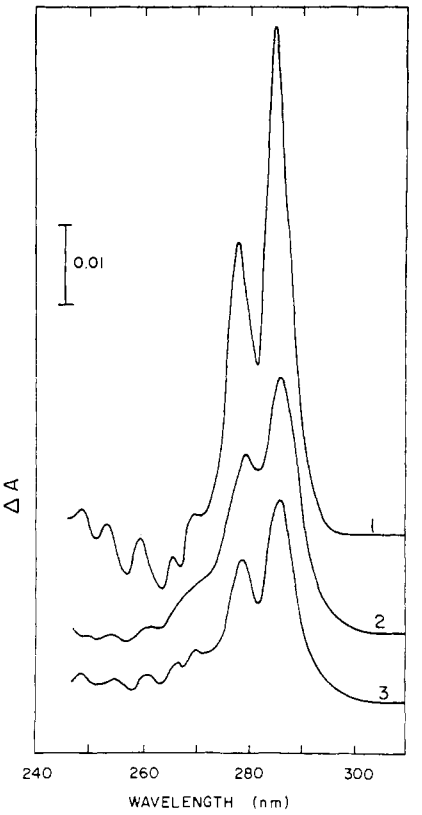

FIGURE 5: Ionic strength and pH difference spectra of zinc insulin. Curve 1: pH 3.5 *vs.* pH 2 as reference, $\mu = 0.01$, 0.096 mg/ml of zinc insulin, 10-cm cells. Curve 2: pH 3.5 *vs.* pH 2 as reference, $\mu = 0.1$, 0.097 mg/ml of zinc insulin, 10-cm cells. Curve 3: $\mu = 0.1$ *vs.* $\mu = 0.01$ as reference, pH 2, 1 mg/ml of zinc insulin, 1-cm cells.

Apparently the presence of zinc ion is not important for the properties of insulin examined in this work. This is expected, since the titration curves of zinc-free and zinc insulin are

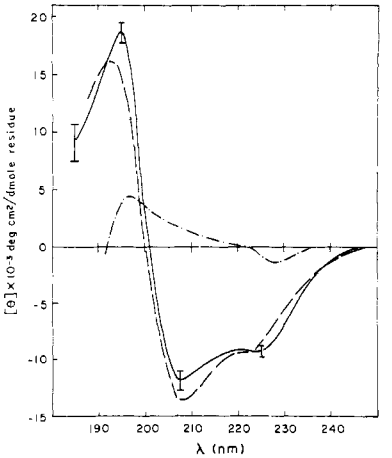

FIGURE 6: CD of zinc insulin in 0.01 M $NaClO_4$ at pH 2 and 1.0 (—) and 0.01 (- - -) mg/ml and the calculated 1.0–0.01-mg/ml difference spectrum (· – ·).

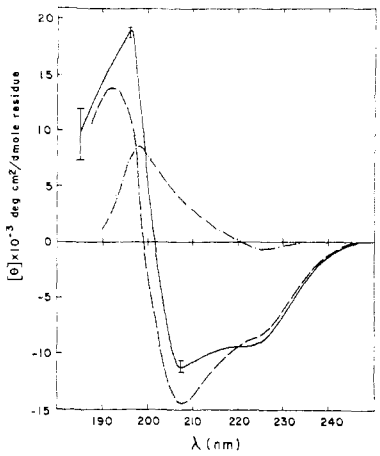

FIGURE 7: CD of zinc insulin in salt-free solution at pH 2 ($HClO_4$) and 1.0 (—) and 0.01 (- - -) mg/ml and the calculated 1.0–0.01-mg/ml difference spectrum (·—·).

virtually identical below pH 4 (Tanford and Epstein, 1954) and zinc ion does not bind to insulin at pH 3.75 (Cunningham *et al.*, 1955).

Discussion

The concentration-dependent absorbance change for insulin comes through association of monomer to dimer and is essentially independent of higher polymer formation. At 25° more than 80% of the change in absorbance occurs between 0 and 2 mg/ml (Figure 2a). Jeffrey and Coates (1966a) fit sedimentation equilibrium data obtained at 25°, pH 2, and 0.1 ionic strength with equilibrium constants that give monomer and dimer as the principal species at low concentration (at 2 mg/ml, about 10% tetramer and almost no hexamer is expected).

Examination of the crystal structure of insulin (Blundell *et al.*, 1971) shows that two tyrosines of the four in the insulin monomer interact significantly with the partner subunit in the dimer. Tyrosine B26, which is partially buried through intramonomer contacts, is almost completely withdrawn from solvent in the dimer (Table II), and tyrosine B16, which is almost completely exposed in the monomer, is largely buried in the dimer. In accord with the absence of a significant spectrum change associated with formation of larger polymers from the dimer, the crystal structure shows that formation of the hexamer results in little further reduction in exposure of tyrosine to solvent. The change in extinction for association of monomer to dimer (480 units) is somewhat larger than the change associated with folding of the chain to the monomer (310 units). It is therefore noteworthy that the total change in tyrosine exposure for chain folding to the monomer is about twice as great as for dimerization. The change in phenylalanine absorbance ($\Delta\epsilon_{260}$) is several times greater for chain folding than for dimerization (70 compared with 25 extinction units; Table I), which accords with the change in exposure (Table II).

The thermodynamic parameters for insulin dimerization at pH 2, 25°, and 0.1 ionic strength calculated from the concentration difference spectrum data are: $\Delta F_u = -8.7$ kcal/mol, $\Delta H^\circ = -17.2$ kcal/mol, and $\Delta S_u = -29$ cal/(degree mol). The large negative value for the enthalpy of association is striking. The origin of such a large enthalpy change is unclear. The crystal structure shows for the dimer seven intersubunit hydrogen bonds and a number of contacts between nonpolar side chains. The latter would be expected to make near zero or small positive contributions to the enthalpy. The formation of seven hydrogen bonds in aqueous solution would not be expected to yield a −17-kcal enthalpy change (Tanford, 1970). It should be noted that in the crystal there are some differences in conformation between the two units that comprise the dimer, and these must develop in the association reaction.

TABLE II: Fractional Exposure of the Chromophoric Side Chains of Insulin to Solvent Relative to Exposure in a Small Peptide Model.[a]

		Monomer	Dimer	Hexamer
Tyr	A14	0.7	0.7	0.5
	A'14	0.7	0.7	0.55
	A19	0.1	0.1	0.1
	A'19	0.3	0.25	0.25
	B16	0.75	0.3	0.25
	B'16	0.75	0.25	0.2
	B26	0.3	0.05	0.05
	B'26	0.35	0.05	0.05
Phe	B1	0.55	0.55	0.05
	B'1	0.6	0.6	0.0
	B24	0.25	0.0	0.0
	B'24	0.15	0.0	0.0
	B25	0.45	0.3	0.3
	B'25	0.65	0.4	0.4

[a] Values for tyrosine and phenylalanine residues in both units (AB and A'B') of the dimer are given. A value of 1 indicates complete exposure to solvent and a value of 0 indicates the chromophore is completely buried. Computations were carried out using a program like that of Lee and Richards (1971) to calculate exposure to solvent from the atomic coordinates kindly supplied by Professor D. C. Hodgkin and her colleagues. The program and the data from which these estimates were made are described by Shrake and Rupley (1973).

The value for K_{12} of 4.0×10^4 M^{-1} for insulin dimerization at 25° and 0.1 ionic strength (Table I) is significantly larger than the value 1×10^4 M^{-1} estimated for the same conditions by Jeffrey and Coates (1966a). The larger association constant found in the present work is likely to be the more accurate value. The sedimentation equilibrium data extend only to about 0.2 mg/ml, conditions under which nearly half the protein is dimer. Sedimentation measurements at 15° should suffer even greater error than at 25°, accounting for the substantially smaller value of the enthalpy of association (−7.1 kcal/mol) obtained by Jeffrey and Coates. The value found for K_{12} in the present experiments is one-third of the value 1.4×10^6 M^{-1} found by Pekar and Frank (1972) using sedimentation equilibrium at pH 7, 25°, and *ca.* 0.1 ionic strength.

The thermodynamic parameters calculated per mole of dimer for the dimer to hexamer reactions were determined by Jeffrey and Coates (1966a) as $\Delta F_u = -4.2$ kcal and $\Delta H^\circ = +11$ kcal. The free energy of the dimer to hexamer reaction is about half that of the monomer to dimer reaction and the enthalpy is of opposite sign. There is no clear difference in the nature of the intersubunit contacts formed in these two processes (Blundell *et al.*, 1971). Interactions between dimers in the hexamer involve 5–10 hydrogen bonds (Blundell *et al.*, 1971), and the extent and character of the interface are similar for dimer and hexamer formation (Shrake and Rupley,

1973). Clearly, the thermodynamics of these association processes must be governed not by the general nature of the contact, such as the extent of the surfaces interacting, but rather by the character of what may be only a small number of highly specific interactions. It is of interest that of the 14 residues in the monomer–monomer interface of the dimer, 6 are conserved in the several insulin species for which sequences have been determined, but of the 12 residues in the dimer–hexamer contact, only 2 are conserved (Blundell *et al.*, 1971).

A change in pH from 2 to 3.5 weakens the monomer–dimer association by about a factor of 1.3 (Table IA). A decrease in ionic strength from 0.1 to 0.01 at constant pH weakens association by a factor of 4. The effect of ionic strength on protein association has been interpreted in terms of electrostatics using Debye–Hückel and other smeared-charge approximations (Jeffreys and Coates, 1966a). Models of this sort are clearly not applicable to insulin dimerization, because a decrease in charge through an increase in pH weakens rather than strengthens the interaction and the effect of ionic strength change is not greatly different at pH 2 and 3.5.

Figure 8 correlates the changes in extinction coefficient for the reactions and transfers of insulin studied in this work. The pH dependence of the insulin spectrum in the acid region reported by Laskowski *et al.* (1960) is confirmed. Under the conditions of their measurements, insulin is predominantly dimer. A similar pH-dependent spectrum change holds for the monomer. Apparently the structural features responsible for this spectrum effect are unaltered by association of the protein. The data of Laskowski *et al.* (1960) show an apparent pK of 3.5 for the ionization (insulin dimer). Because K_{12} is slightly smaller at pH 3.5 than pH 2, the ionization in the monomer would have pK $\simeq$3.4. Thus the total spectrum change associated with complete deprotonation would be *ca.* 340 extinction units at 286 nm for the monomer and 420 extinction units for the dimer. These values compare well with the estimate of 370 units from the data of Laskowski *et al.* (1960). It is of interest that the size of the pH-dependent spectrum change is comparable to that measured for monomer association and for folding of the chain. There should be no significant change in exposure of tyrosines to solvent over this pH range (2–4). Withdrawal from solvent can be only one of the significant perturbants of the insulin chromophores. The lack of correlation between change in exposure and tyrosine perturbation was noted above for comparison of chain folding and association.

The spectrum change associated with dimerization at pH 2 and 0.01 ionic strength is slightly larger than that measured at 0.1 ionic strength. This reflects the greater effect of change in ionic strength on the spectrum of the monomer compared with the dimer. There are no data that define the spectrophotometrically operable pK at ionic strength 0.01. The weaker association of monomer to dimer at pH 3.5 compared with pH 2, however, requires that the pH-dependent spectrum changes for monomer and dimer be more nearly equal for full deprotonation than is indicated for the partial protonation reactions of Figure 8. Correspondingly, the extinction changes for conversion of monomer to dimer must be more nearly equal at low ionic strength if the conversions between states of full deprotonation and full protonation are considered.

The wavelengths of the difference spectra maxima measured for the various reactions of insulin range from 285.0 to 288.5 nm (Table I). This is not surprising considering that insulin has four tyrosines and that different residues are perturbed by the various processes of Table I. For example, examination of the crystal structure shows that the residue most

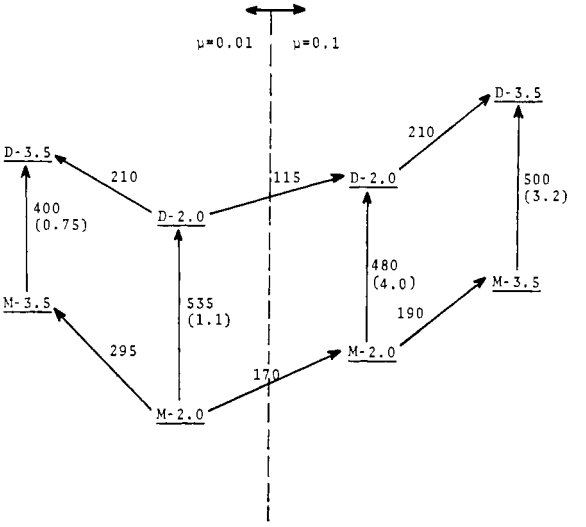

FIGURE 8: Correlation of change in extinction coefficient for changes in state of insulin. Values in parentheses are association constants.

strongly affected by folding (tyrosine A19; Table II) is not affected by conversion of monomer to dimer. Tyrosine B26, on the other hand, is little affected by folding but is strongly involved in dimerization. Also, the effects of pH, ionic strength, and dimerization on the spectrum apparently are relatively independent (Table I and Figure 8).

Disruption of ordered structure on dissociation of the dimer is indicated by the change in the CD spectrum on dilution. The shape of the difference CD spectrum suggests that more β structure is present in the dimer than in the monomer. Such a conformation is part of the monomer–monomer interface seen in the crystal structure (Blundell *et al.*, 1971), and it was suggested by Ettinger and Timasheff (1971a,b) to explain the anomalous position of the positive CD extremum at 196 nm and the changes with solvent composition. Assuming only four residues per dimer are involved in intersubunit β structure as seen in the crystal, the magnitude of the difference at 197 nm found on dilution (6000–10,000 deg cm^2/dmol of residue for conversion of monomer entirely to dimer) is too large to be accounted for on the basis of known polypeptide models. Additional contributions, particularly from tyrosine and phenylalanine transitions, are likely. In this connection, the near-ultraviolet CD has been shown to be aggregation dependent by Morris *et al.* (1968) and by Carpenter and Hayes (1966).

The larger CD changes found for dilution in salt-free solution compared with the changes in 0.01 M $NaClO_4$ indicate that the environments of some elements of the insulin dimer or monomer are dependent on ionic strength as well as aggregation. In this regard, the phenylalanine perturbations listed in Table I show that for the insulin monomer relatively large changes in $\Delta\epsilon$ are produced by changes in ionic strength.

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Conformational Analysis of the Polypeptide Antibiotic Telomycin by Nuclear Magnetic Resonance†

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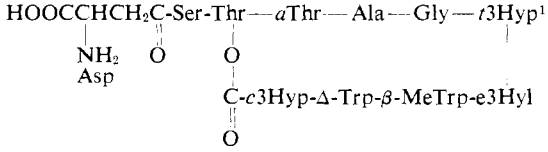
ABSTRACT: The solution conformation of the polypeptide antibiotic telomycin in Me_2SO-d_6 has been studied through the use of 220-MHz ^1H nuclear magnetic resonance (nmr) spectroscopy. In addition to the information provided by chemical shifts and the vicinal α-CH–NH coupling constants in elucidating conformation, three methods—proton–deuteron exchange, temperature dependence of peptide proton chemical shift, and methanol–trifluoroethanol solvent mixture dependence of peptide proton chemical shift—are used to delineate peptide protons in terms of exposure to solvent. All three methods clearly define the threonine, hydroxyleucine, and β-methyltryptophan peptide protons as solvent shielded. These data are utilized in discussing a conformation of telomycin in which there are three hydrogen-bonded rings of ten atoms, two of which are typical β turns.

Telomycin, an undecapeptide antibiotic, was isolated from the culture broth of an unidentified streptomyces by Misiek *et al.* (1957–1958). Its primary structure was determined by Sheehan *et al.* (1963, 1968).



It is the purpose of this work to present proton magnetic resonance (pmr) results on the conformational aspects of telomycin. The spectral features for distinguishing between solvent-exposed and solvent-shielded peptide protons (Kopple *et al.*, 1969; Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Pitner and Urry, 1972a) are discussed as they apply to telomycin. Detailed discussion of the solution conformation compatible with the experimental nmr data is given.

Experimental Section

Pmr spectra were recorded on a Varian Associate HR-220 spectrometer, as is described elsewhere (Kumar and Urry, 1973). The chemical shift difference between resonances of ethylene glycol or methanol was used to determine the probe temperature. Chemical shifts were measured relative to tetramethylsilane as an internal reference, unless otherwise indicated. Telomycin was shown to be homogeneous by thin-layer chromatography (Kumar and Urry, 1973).

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[1] Abbreviations used are: *trans*-3Hyp and *cis*-3Hyp, *trans*- and *cis*-3-hydroxyproline, respectively; Δ-Trp, α,β-didehydrotryptophan; e3Hyl, *erythro*-3-hydroxyleucine. All amino acids in telomycin are reported to be of the L configuration (Sheehan *et al.*, 1963, 1968).