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Self-Association of Insulin and the Role of Hydrophobic Bonding: A Thermodynamic Model of Insulin Dimerization†

Y. Pocker* and Subhasis B. Biswas

ABSTRACT: The association constant for insulin dimerization calculated from concentration-dependent circular dichroic (CD) spectra of insulin, $K_{12} = 7.5 \times 10^5$ M^{-1}, is used along with other association constants (K_{24}, K_{46}, and K_{26}) in an attempt to decipher the complex association behavior in solution and in crystal of this protein hormone. The free-energy change associated with dimerization, $-RT \ln K_{12}$, is -8.01 kcal mol^{-1}, a value which is used to test a semiquantitative thermodynamic model of the process based in part on the X-ray crystallographic data of insulin. By delineation of the hydrophobic core on the surface of insulin, which is implicated in the dimer formation, the free energy of association, $\Delta G°_{assoc}$, is estimated as -8.27 kcal mol^{-1} by using the thermodynamic parameters of Némethy & Scheraga [Némethy, G., & Scheraga, H. A. (1962) *J. Phys. Chem. 66*, 1773-1789] and as -10.27 kcal mol^{-1} by using the values of Nozaki & Tanford [Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211-2217]. The role of hydrophobic bonding in the dimerization of insulin is discussed, and the calculated values of free energy of association are compared with the experimental value. The importance of this thermodynamic model is delineated in regard to both hormone-hormone and hormone-receptor interactions.

Insulin exhibits a complex association behavior in solution and in crystal (Jeffrey & Coates, 1966; Pekar & Frank, 1972; Lord et al., 1973; Goldman & Carpenter, 1974; Pocker & Biswas, 1980). A monomer-dimer-tetramer-hexamer (M-D-T-H)[1] equilibria for insulin solutions were proposed by

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[1] Abbreviations used: CD, circular dichroism; M-D-T-H, monomer-dimer-tetramer-hexamer; M-D-H, monomer-dimer-hexamer; assoc, association; ent, entropic.

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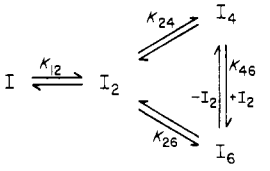
Scheme I



Jeffrey & Coates (1966), Goldman (1971), and Goldman & Carpenter (1974) on the basis of their equilibrium sedimentation studies. On the other hand, Pekar & Frank (1972) proposed a simpler monomer–dimer–hexamer (M–D–H) scheme. Therefore, the association behavior of insulin can be generalized as in Scheme I.

Different values of K_{12}, K_{26}, K_{24}, and K_{46} are reported in the literature. The variations in the reported values of the association constants are due in part to differences in experimental conditions, e.g., pH, ionic strength, and metal ion (Zn^{2+}) concentration, and in part to experimental and computational errors.

In our analysis, we calculated K_{12} from the concentration-dependent circular dichroic[1] spectra of insulin (Figure 1) and K_{24} and K_{46} from the apparent weight average molecular weight ($M_{w,app}$) data of Goldman & Carpenter (1974). The values of $M_{w,app}$ at various concentrations calculated for the reported K_{12}, K_{24}, and K_{46} or K_{26} constants are plotted against concentration, and the theoretical plots are compared with the experimental $M_{w,app}$ vs. C plot of Goldman & Carpenter in order to determine the best set of values of the association constants.

In the last few years, there has been much speculation on the modes of interaction between two insulin monomers to form the dimer. Various experimental and X-ray crystallographic data unequivocally establish that the residues Phe and Tyr, present in B-chain C-terminal, and other hydrophobic groups such as Val, Tyr, etc., of insulin play an important role in the dimerization process and in the stability of the dimer. The groups involved in this process are nonpolar; consequently the dimerization must proceed essentially through hydrophobic interactions between nonpolar groups of the two monomers involved. The X-ray crystallographic data also reveal that the nonpolar groups A_{19} Tyr, B_{12} Val, B_{16} Tyr, B_{24} Phe, B_{25} Phe, and B_{26} Tyr form a "hydrophobic core" on the surface of each monomer (Pullen et al., 1976; De Meyts et al., 1978; Blundell et al., 1972). This hydrophobic core is involved not only in the self-association process but also probably in the hormone–receptor interaction. Earlier observations such as the complete dissociation of insulin in 40% dioxane (Fredericq, 1953, 1956) and the dependence of insulin association on pH and ionic strength (Fredericq, 1953, 1956; Fredericq & Neurath, 1950; Pocker & Biswas, 1980) indicate that hydrophobic bonds[2] are primarily involved in insulin dimerization. Earlier, Némethy & Scheraga (1962) and Scheraga (1979) treated quantitatively the thermodynamic properties of hydrophobic bonds formed by close contacts of two or more nonpolar side chains in a protein by using aqueous hydrocarbon solutions as model systems following the principles of hydrophobic bonding originally formulated by Kauzmann (1959). They calculated thermodynamic parameters of hydrophobic bonds involving nonpolar amino acid residues and proposed

[2] We are tentatively using the terms "hydrophobic bond" and "hydrophobic interaction" for want of better names. Certain authors regard both terms as somewhat misleading: the former does not refer to a chemical bond in the ordinary sense, while the latter does not properly specify the mechanism of interaction.

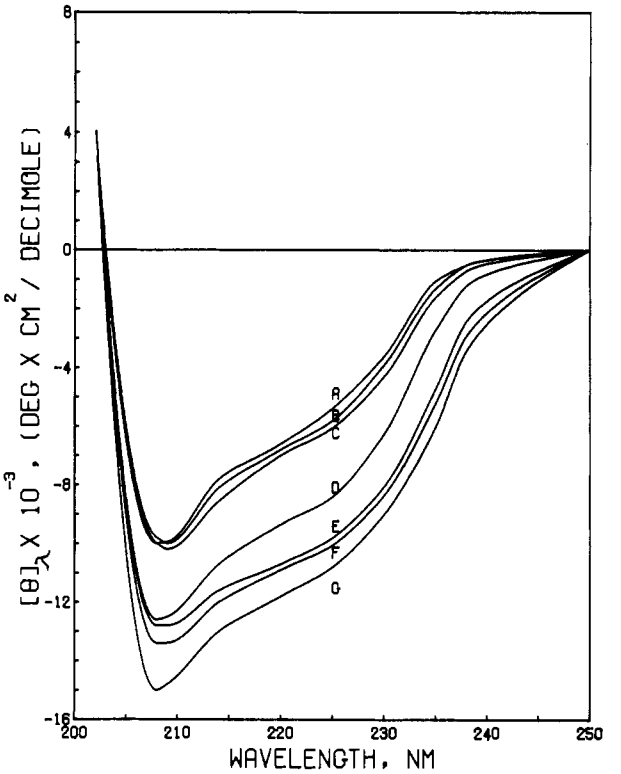


FIGURE 1: The experimental and computed far-ultraviolet circular dichroic spectra of insulin in solution. The spectra (B–F) are the experimental spectra of Zn-free bovine insulin at concentrations 100 nM (B), 250 nM (C), 500 nM (D), 1 μM (E), and 100 μM (F). The measurements were carried out in 0.005 M phosphate buffers of pH 7.0 and at 25 °C. All spectra were the average of 3–9 scans, and the estimated errors in measurements are indicated in Table I. The computed spectra of insulin in monomeric and dimeric states are A and G, respectively. These are computed from a plot of $[\theta]_\lambda$ vs. $f_{monomer}$ and extrapolated to $f_{monomer} = 1.0$ for the spectra of monomer and extrapolated to $f_{monomer} = 0$ for the spectra of dimer. Measurements were at very low concentrations so that a true monomer–dimer equilibrium exists (cf. Pocker & Biswas, 1980).

a set of values which appeared to us to be reliable enough for a semiquantitative treatment of protein association through hydrophobic bonding.

In this paper, we use these thermodynamic parameters along with a somewhat modified derivation of the entropy of association of proteins (Steinberg & Scheraga, 1963) to formulate a semiquantitative thermodynamic model of insulin dimerization. The free energy of association, calculated by using this model, is −8.27 kcal mol^{-1}, a value which is in good agreement with the experimental free energy of association, −8.01 kcal mol^{-1}, calculated by using an association constant K_{12} of 7.5×10^5 M^{-1}. The possibility of examining the validity of such semiquantitative thermodynamic models, despite the approximations made in course of their formulation, is encouraging, especially in regard to studies pertaining to protein–protein and hormone–receptor interactions.

Experimental Procedures

Materials. Zn-insulin was purchased from Sigma Chemical Co. (lot no. 47C-0264) and was also obtained as a gift from both Dr. Ronald E. Chance of Lilly Research Laboratories and Dr. E. Schlichtkrull of NOVO Research Institute, Denmark. Zn-free insulin hydrochloride was prepared by the method of Goldman & Carpenter (1974), and purified by gel filtration chromatography.

Measurements. Insulin solutions were prepared in 0.005 M phosphate buffer by using deionized redistilled water and reagent grade KH_2PO_4 and K_2HPO_4. A Cary 14 spectro-

photometer was used to determine the concentration of various insulin solutions. All CD spectra were recorded on an ultrasensitive Jobin Yvon Dichrograph Mark III. The dichrograph was calibrated by using 0.002 M *d*-10-camphorsulfonic acid (Eastman Kodak Co.) which was purified by recrystallization from hot acetic acid followed by sublimation. All spectra were recorded at pH 7.0 and 25 °C. The procedures for recording CD spectra and calculating mean residue ellipticities are identical with those used previously (Pocker & Biswas, 1980).

Computation of K_{12}, K_{24}, and K_{46}. For a system of interacting proteins, which exhibits differences in circular dichroic spectra of each component protein, the association constants can be evaluated from an analysis of circular dichroic spectra. At lower concentrations, insulin is involved in a monomer–dimer equilibrium (Goldman & Carpenter, 1974; Pocker & Biswas, 1980). For such a system, the observed mean residue ellipticity, $[\theta]_\lambda$, at any wavelength λ can be expressed in terms of eq 1 where "M" refers to monomer, "D" refers to dimer,

$$[\theta]_\lambda = f_M[\theta]_M + f_D[\theta]_D + \beta C \quad (1)$$

"f" stands for fraction of each species at equilibrium, and "β" is the second virial coefficient arising from the nonideality of the protein solution. At very low concentrations, the magnitude of the second virial coefficient is small and can be neglected. Hence, eq 1 reduced to eq 2.

$$[\theta]_\lambda = f_M[\theta]_M + f_D[\theta]_D \quad (2)$$

The sum of the two fractions is unity, $f_M + f_D = 1$ or $f_D = 1 - f_M$, and therfore $[\theta]_\lambda = f_M([\theta]_M - [\theta]_D) + [\theta]_D$, leading to eq 3.

$$f_M = \frac{[\theta]_D - [\theta]_\lambda}{[\theta]_D - [\theta]_M} \quad (3)$$

The values of $[\theta]_M$ and $[\theta]_D$ at $\lambda = 223$ nm were calculated from the theoretical CD spectra of monomeric and dimeric insulin as shown in Figure 1 (cf. Pocker & Biswas, 1980).

If the total concentration of the protein in terms of monomer is C, then K_{12} can be expressed in terms of

$$K_{12} = \frac{1 - f_M}{2f_M^2}C^{-1}$$

By use of eq 3 for f_M, the association constant will be

$$K_{12} = \left(\frac{[\theta]_\lambda - [\theta]_M}{[\theta]_D - [\theta]_M}\right) \Big/ \left[2C\left(\frac{[\theta]_D - [\theta]_\lambda}{[\theta]_D - [\theta]_M}\right)^2\right] \quad (4)$$

The apparent weight average molecular weight of insulin, $M_{w,app}$, can be defined in terms of eq 5 where $i = 1, 2, 4$, and 6, Table I.

$$M_{w,app} = \sum C_i M_i / \sum C_i \quad (5)$$

Two different models of insulin association are proposed in Scheme I. If C_1 is the concentration of monomer and C_n is the concentration of n-mer, the concentrations of other states can be calculated for the models displayed in Scheme I by using (M–D–T–H and M–D–H models, respectively) $2K_{12}C_1^2$ and $2K_{12}C_1^2$ for C_2, $4K_{24}K_{12}^2C_1^4$ and 0 for C_4, and $6K_{46}K_{24}K_{12}^3C_1^6$ and $6K_{26}K_{12}^3C_1^6$ for C_6, where C_2, C_4, and C_6 are the concentrations of dimer, tetramer, and hexamer, respectively, expressed in terms of monomeric concentrations and K_{12}, K_{24}, K_{46}, and K_{26} are the association constants as defined in Scheme I. Therefore the total concentration of insulin can be expressed by eq 6a (M–D–T–H model) and 6b (M–D–H model). These equations were used to compute K_{24}

$$C = C_1 + 2K_{12}C_1^2 + 4K_{24}K_{12}^2C_1^4 + 6K_{46}K_{24}K_{12}^3C_1^6 \quad (6a)$$

$$C = C_1 + 2K_{12}C_1^2 + 6K_{26}K_{12}^3C_1^6 \quad (6b)$$

Table I: Circular Dichroic and Association Parameters of Insulin at Various Concentrations at pH 7.0 and 25 °C

concn (μM)	$-[\theta]_{223} \times 10^{-3}$ [a] (deg cm^2 dmol^{-1})	$f_{monomer}$ [c]	$M_{w,app}$ [b]	av [d] $K_{12} \times 10^{-5}$ (M^{-1})
10	10.3 ± 0.2	0.191	10 500	
5	9.9 ± 0.2	0.267	10 200	
1	8.8 ± 0.3	0.478	7 800	
0.75	8.3 ± 0.4	0.574	7 600	7.5
0.50	7.8 ± 0.4	0.669	7 000	
0.25	6.7 ± 0.6	0.879	6 200	
0.10	6.6 ± 0.8	0.889	5 950	

[a] $[\theta]_{223}$ is the mean residue ellipticity of insulin at 223 nm. The value of $[\theta]_{223}$ is the average of 3–9 scans, and the estimated error in measurements at each concentration is as indicated. [b] $M_{w,app}$ is the apparent weight-average molecular weight of insulin at various concentrations at pH 8.0 and 25 °C. The values are obtained from the $M_{w,app}$ vs. C plot of Goldman (1971) and Goldman & Carpenter (1974). For details of experimental conditions, see Goldman & Carpenter (1974). [c] $f_{monomer}$ is the fraction of the total amount of insulin in monomeric state, which is calculated from $[\theta]_{223}$ at corresponding concentrations. [d] K_{12}, the association constant for insulin dimerization, is calculated from $[\theta]_{223}$ by using eq 4 at very silute solutions where true monomer–dimer equilibrium exists. For details of calculation, see text.

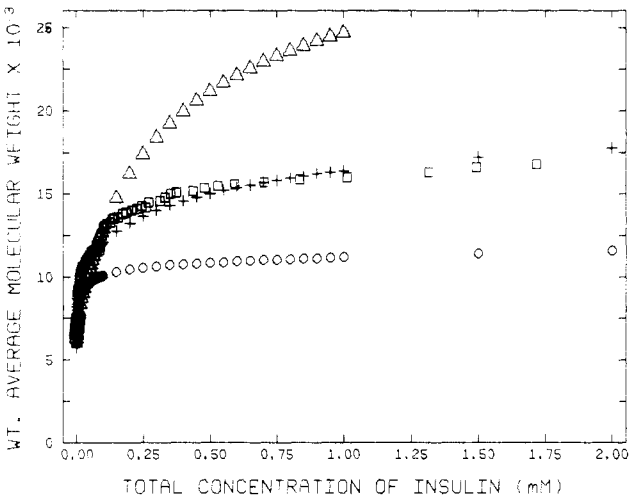


FIGURE 2: The computed and experimental plots of apparent weight average molecular weight ($M_{w,app}$) vs. total concentration of insulin in terms of monomer. The plots are based on the associatibn constants of Pekar & Frank (1972) (△), Goldman & Carpenter (1974) (○), this report (+), and experimental data of Goldman & Carpenter (□). Details of computation are presented under Experimental Procedures. For experimental conditions, see references.

and K_{46}. The value of K_{12} was available from CD studies. We computed C_1, C_2, C_4, C_6, and $M_{w,app}$ by using a program developed locally by Dr. C. T. O. Fong, which employs least-squares iterative techniques, and the resulting plot of $M_{w,app}$ vs. C was compared with the experimental plot of Goldman & Carpenter (1974). The values of K_{24} and K_{46} were varied until a reasonable agreement between the theoretical and experimental plots was achieved at all protein concentrations (Figure 2). During such analysis, we observed that the M–D–H model does not produce a plot of $M_{w,app}$ vs. C, which is in reasonable agreement with the experimental plot of Goldman & Carpenter for any values of K_{26} and consequently are predisposed toward the M–D–T–H model of insulin association. Parallel computations were also carried out on a CDC 6400 computer using a ZPOLR program (IMSL Library). Both programs generated the same values of K_{24} and K_{46}. These are presented in Table II and compared with the values reported by earlier workers. Small variations in K_{24} and K_{46}

Table II: Reported Association Constants of Insulin

pH	ionic strength	K_{12}	K_{24}	K_{46}	K_{26}	ref[a,b]
2.0	0.10	1.25×10^4	7.8×10^2	6.7×10^2		1
8.0	0.10	2.22×10^5	40	220		2
7.0		1.40×10^5			4×10^8	3
2.0		4.00×10^4				4
7.0		7.50×10^5	5.0×10^3	45		5

[a] (1) Jeffrey & Coates (1966); (2) Goldman (1971) and Goldman & Carpenter (1974); (3) Pekar & Frank (1972); (4) Lord et al. (1973); (5) present work. [b] It is well-known that the association equillibria of insulin depend intimately on pH, ionic strength, etc. The association constants reported by various groups, as indicated, were determined under various physical conditions; therefore, the variations in the reported values of the association constants are not unexpected.

could arise from differences in pH and ionic strength. Reported association constants were analyzed by using eq 6, and we used a computer program written by Dr. C. T. O. Fong for our PDP 8/E computer which is interfaced with a Calcomp plotter. Each set of association constants (Pekar & Frank, 1972; Goldman & Carpenter, 1974; this report) was used to generate a plot of $M_{w,app}$ vs. C, and those three plots are presented in Figure 2 together with the experimental plot of Goldman & Carpenter. The values of K_{12} reported by previous workers are comparable to those reported here. The differences in the values of K_{24} and K_{46} are probably due to computational error and slight differences in experimental condition such as pH and ionic strength which influence this association of insulin. However, Figure 2 clearly demonstrates the constants reported here provided the best fit for the experimental plot. A theoretical distribution of each associated species has been computed in a PDP 8/E computer and replotted in Figure 4.

Estimation of the Free Energy of Association. X-ray crystallographic investigations (Blundell et al., 1971, 1972; Peking Insulin Group, 1974; Dodson et al., 1979) have established the three-dimensional structure of insulin hexamers in crystal. A critical analysis of these reports reveals a rather unique feature of the insulin dimer in crystal. Apparently, several hydrophobic side chains (listed in Table III) come close together and form a hydrophobic core on the surface of insulin. These side chains have considerable flexibility and therefore facilitate the formation of the core by making the necessary alterations in their three-dimensional orientation which are evident from the apparent dissymmetry in the insulin dimer. On the basis of these observations and the fact that the removal of the hydrophobic residues from the B-chain C-terminal of insulin greatly reduces its ability to dimerize, we attempted to develop a thermodynamic model of insulin dimerization.

In order to compute the free energy of association ($\Delta G°_{assoc}$) of two proteins, it is necessary to consider (i) the contribution of hydrophobic interactions between the proteins involved and the hydrophobic interactions between nonpolar groups in each individual protein involved, (ii) the contribution of translational, rotational, and vibrational entropies due to association, and (iii) the contribution of conformational changes of each protein molecule during association.

Consider a protein association that involves in the initial state n protein molecules, each of which has several intramolecular hydrophobic interactions (m), and in the final state of association, forms several intermolecular hydrophobic bonds (l). The free energy of association of such a system can be expressed by

$$\Delta G°_{assoc} = \sum_{i=1}^{l}(C_i \delta G°_i) - \sum_{k=1}^{n}[\sum_{j=1}^{m}(C_{kj} \delta G°_{kj})] + \Delta G°_{ent} \quad (7)$$

where $\Delta G°_{ent}$ is the intrinsic entropic change due to dimerization. This generalized expression for $\Delta S°_{assoc}$ can be used to estimate the free energy of dimerization of insulin.

Table III: Minimum Contact Distances between Nonpolar Side Chains at the Dimer Interface[a]

side chain[b]	side chain[b]	contact distance (Å)
A_{19} Tyr I	B_{25} Phe I	3.765
B_{12} Val I	B_{26} Tyr I	4.034
	B_{12} Val II	3.972
	B_{16} Tyr II	2.917
B_{16} Tyr I	B_{24} Phe I	4.074
	B_{25} Phe I	4.576
	B_{12} Val II	4.095
B_{24} Phe I	B_{25} Phe I	3.624
	B_{26} Tyr I	4.933
	B_{24} Phe II	3.665
	B_{26} Tyr II	3.558
B_{25} Phe I	B_{24} Phe I	3.624
	B_{25} Phe II	3.079
B_{26} Tyr I	B_{16} Tyr II	3.603
	B_{24} Phe II	2.993
A_{19} Tyr II	B_{25} Phe II	3.536
B_{12} Val II	B_{24} Phe I	3.812
	B_{24} Phe II	4.435
	A_{26} Tyr II	3.659
B_{16} Tyr II	B_{26} Tyr II	3.603
B_{24} Phe II	B_{12} Val I	4.798
	B_{16} Tyr II	4.669
	B_{26} Tyr II	3.970
B_{25} Phe II	A_{19} Tyr II	4.400
B_{26} Tyr II	B_{12} Val I	3.659
	B_{16} Tyr I	4.061
	B_{25} Phe II	3.640

[a] These calculated contact distances are obtained by a computer analysis (program XRAY-76) of the X-ray coordinates of the atoms of insulin. All contact distances above 5 Å and covalent bonds were ignored in these calculations. By examination of the atom to atom distances of two side chains, the groups in possible contact were chosen and reported here. [b] I and II refer to monomer I and monomer II, respectively, in the dimer.

Accordingly, this process involves primarily hydrophobic interactions between nonpolar side chains, i.e., tyrosine, phenylalanine, and valine. Némethy & Scheraga (1962) calculated the free energy of hydrophobic interaction between various side chains in a protein, and the calculated thermodynamic parameters are correlated with experimental values; therefore, these parameters are suitable for estimating free energies of protein interaction. The expression for $\Delta G°_{assoc}$ is presented in eq 7. The values of $\delta G°_i$ and $\delta G°_{kj}$ were evaluated from Némethy & Scheraga (1962) and Nozaki & Tanford (1971) and are given in Tables IV and V. The coefficients of interaction C_i and C_{kj} can be evaluated only by detailed computer modelling of the available X-ray crystallographic data. In the present paper, we assigned a maximum value of 1.0 to all C_i and C_{kj} terms. The term $\Delta G°_{ent}$ in eq 7 represents the change in free energy due to loss of degrees of freedom during the formation of the dimer from monomers and can be calculated by

$$\Delta G°_{ent} = -T\Delta S° = -T(\Delta S°_{tr} + \Delta S°_{rot} + \Delta S°_{vib}) \quad (8)$$

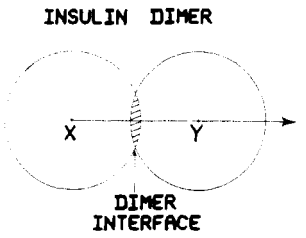


FIGURE 3: A diagram of the insulin dimer where each monomer was considered as a solid sphere. Each insulin molecule contains a unimolecular layer of water molecules with an effective radius of 14.2 Å. The dashed region represents the hydrophobic core in the dimer where the hydrophobic groups of both molecules are in contact.

Table IV: Contributions of Various Residues to the Thermodynamic Parameters in the Dimerization of Insulin[a]

contributing residues[b]	δG°_i[c] (kcal mol^{-1})	δH°_i[c] (kcal mol^{-1})	δS°_i[c] (eu)	$-T\delta S^\circ_i$[c] (kcal mol^{-1})	δG°_i[d] (kcal mol^{-1})
A_{19} Tyr I	−1.2	+0.7	+6.1	−1.82	−2.3
B_{12} Val I	−1.9	+2.2	+13.7	−4.08	−1.5
B_{16} Tyr I	−1.2	+0.7	+6.1	−1.82	−2.3
B_{24} Phe I	−1.8	+1.0	+9.5	−2.83	−2.5
B_{25} Phe I	−1.8	+1.0	+9.5	−2.83	−2.5
B_{26} Tyr I	−1.2	+0.7	+6.1	−1.82	−2.3
A_{19} Tyr II	−1.2	+0.7	+6.1	−1.82	−2.3
B_{12} Val II	−1.9	+2.2	+13.7	−4.08	−1.5
B_{16} Tyr II	−1.2	+0.7	+6.1	−1.82	−2.3
B_{24} Phe II	−1.8	+1.0	+9.5	−2.83	−2.5
B_{25} Phe II	−1.8	+1.0	+9.5	−2.83	−2.5
B_{26} Tyr II	−1.2	+0.7	+6.1	−1.82	−2.3
total	−18.2	+12.6	+102.0	−30.40	−26.8

[a] Reported values of Nemethy & Scheraga (1962) and Scheraga (1979). [b] I and II refer to monomer I and monomer II, respectively. X-ray crystallographic data, presented in Table III, indicate that the side chains of these residues are involved in the intermolecular contacts between two monomers in the insulin dimer. [c] We noted a small error due to rounding off in the values of δG°_i, δH°_i, and δS°_i. [d] Reported values of Nozaki & Tanford (1971) and Tanford (1970).

where ΔS°_{tr}, ΔS°_{rot}, and ΔS°_{vib} are the changes in standard entropy due to loss of translational, rotational, and vibrational degrees of freedom, respectively.

The translational contribution to the intrinsic entropy of dimerization can be derived from the Sackur–Tetrode equation if the contribution of solvent molecules to the partial molar entropy of the dimer and the monomers is neglected. The expression for ΔS°_{tr} is then given by

$$\Delta S^\circ_{tr} = R\left[\ln\left(\frac{N}{V}\left(\frac{h^2}{\pi nkT}\right)^{3/2}\right) - 3/2\right] \quad (9)$$

The contribution of the solvent molecules to the ΔS°_{tr} can be expressed as $(\bar{S}_{dimer} - 2\bar{S}_{monomer})$. In a case such as the dimerization of insulin where association is due to hydrophobic interactions, the contribution of solvent molecules is significant. But this contribution of the solvent molecules has been accounted for already in the first term of the expression for ΔG°_{assoc}, $\sum_{i=1}^{I}(C_i\delta G^\circ_i)$ because the expression for δG°_i is $\delta H^\circ_i - T\delta S^\circ_i$.

Two insulin molecules associate at C-terminal of B chain. Therefore, a small fraction of the surface is available for association, and the dimer will be a fairly rigid molecule with two spheres of radius 14.2 Å (including a layer of water molecules attached to the protein molecule) as shown in Figure 3. Steinberg & Scheraga (1963) showed that for such a system the rotational contribution to the intrinsic entropy of the system can be expressed in terms of eq 10 where I represents the moment of inertia, α is the fraction of the total protein surface involved in the dimer formation, and β is the fraction of total rotation permitted around the axis (x,y) joining the center of gravity of the two molecules. We calculated α to be 0.065 from the X-ray structure of insulin and β to be 1.0 for this particular case.

$$\Delta S^\circ_{rot} = R \ln \frac{8\pi^2 IkT}{h^2} + R \ln (\alpha^2\beta) + 1 \quad (10)$$

The vibrational contribution to the entropy of such a system can be expressed in terms of eq 11

$$\Delta S^\circ_{vib} = R \ln \frac{kT}{h\nu} + R \quad (11)$$

where the frequency, ν, can be expressed in terms of ΔU_{max}, the bond energy, n, the mass of each particle, and ΔX_{max}, the maximum amplitude of vibration, as follows

$$\nu = \frac{1}{\pi \Delta X_{max}}\sqrt{\frac{\Delta U_{max}}{n}}$$

Introducing the expressions for ΔS°_{tr}, ΔS°_{rot}, and ΔS°_{vib} in the expression for ΔG°_{ent}, we obtain eq 12.

$$\Delta G^\circ_{ent} = -T\left[R \ln\left(\frac{N}{V}\left(\frac{h^2}{\pi nkT}\right)^{3/2}\right) + R \ln \frac{8\pi^2 IkT}{h^2} + R \ln (\alpha^2\beta) + R\left(\ln \frac{kT}{h\nu}\right) + R - 1/2\right] \quad (12)$$

By use of $M_w/N = n$, where M_w is the molecular weight and N is Avogadro's number, the entity ΔG°_{ent} can be expressed in terms of eq 13.

$$\Delta G^\circ_{ent} = -T[73.987 - 2.3 \log T - 2.3 \log M_w] + [9.2 \log r + 4.58 \log (\alpha^2\beta) + 4.58 \log (kT/h\nu)] \quad (13)$$

For the case of insulin dimer, we follow a slight modification of the method of Steinberg & Scheraga (1963) and tentatively estimate ν as equal to 2.46×10^{11}, and $kT/h\nu = 25.20$. By

Table V: Thermodynamic Parameters of Hydrophobic Interactions in Monomer I and Monomer II

subunit	contributing residues	δG°_{kj}[a] (kcal mol^{-1})	δH°_{kj}[a] (kcal mol^{-1})	δS°_{kj}[a] (eu)	δG°_{kj}[b] (kcal mol^{-1})
monomer I	A_{19} Tyr	−1.2	+0.7	+6.1	−2.3
	B_{24} Phe–B_{25} Phe	−1.4	+0.8	+7.5	−2.5
	B_{26} Tyr	−1.2	+0.7	+6.1	−2.3
monomer II	A_{19} Tyr	−1.2	+0.7	+6.1	−2.3
	B_{24} Phe–B_{25} Phe	−1.4	+0.8	+7.5	−2.5
	B_{26} Tyr	−1.2	+0.7	+6.1	−2.3
total		−7.6	+4.4	+39.4	−14.2

[a] Reported values of Némethy & Scheraga (1962) and Scheraga (1979). [b] Reported values of Nozaki & Tanford (1971) and Tanford (1970).

Table VI: Thermodynamic Parameters for the Dimerization of Insulin

thermodynamic parameters[a]	estimated values (kcal mol^{-1}) b	c
$+\Sigma^{l}_{i=1}(C_i\,\delta G^{\circ}{}_i)$	−18.2	−26.8
$-\Sigma^{2}_{k=1}\,\Sigma^{m}_{j=1}(C_{kj}\,\delta G^{\circ}{}_{kj})$	+7.6	+14.2
$+\Delta G^{\circ}{}_{ent}$	+2.33	+2.33
$\Delta G^{\circ}{}_{assoc}$	−8.27	−10.27

[a] Parameters involved in eq 8 and also in eq 14. [b] δG° from Némethy & Scheraga (1962) and Scheraga (1979). [c] δG° from Nozaki & Tanford (1971).

use of 298 K for T and 14.2 Å for r, we obtain for $\Delta G^{\circ}{}_{ent}$ the value of +2.33 kcal mol^{-1}. In short, $\Delta G^{\circ}{}_{assoc}$ can be expressed in terms of eq 14, specifically for insulin dimerization. The

$$\Delta G^{\circ}{}_{assoc} = \sum_{i=1}^{l}(C_i\delta G^{\circ}{}_i) - \sum_{k=1}^{n}\left(\sum_{j=1}^{m}(C_{kj}\delta G^{\circ}{}_{kj})\right) - T\left[R\ln\left(\frac{N}{V}\left(\frac{h^2}{\pi nkT}\right)^{3/2}\right) + R\ln\frac{8\pi^2 IKT}{h^2} + R\ln(\alpha^2\beta) + R\ln\frac{kT}{h\nu} + R - \tfrac{1}{2}\right] \quad (14)$$

thermodynamic parameters involved in eq 7 and 14 are computed for insulin dimerization and presented in Table VI. In the derivation of eq 7 and 14, we tentatively ignored the value of $\Delta G^{\circ}{}_{cc}$, the change of free energy arising from the conformational change.

Results and Discussion

Association Constants. The fact that insulin is a protein hormone of profound physiological significance makes its dynamics in solution an attractive subject of in-depth studies. The association behavior of insulin has been studied in several laboratories (Fredericq & Neurath, 1950; Jeffrey & Coats, 1965, 1966; Pekar & Frank, 1972; Lord et al., 1973; Goldman & Carpenter, 1974). The most widely accepted model of insulin association is monomer ⇌ dimer ⇌ tetramer ⇌ hexamer. The reported association constants K_{12}, K_{24}, and K_{46} vary, depending on the methods and physical conditions of measurement. We calculated K_{12} (monomer–dimer equilibrium constant) from our concentration-dependent circular dichroic studies (Figure 1). We showed earlier that the CD spectra of monomer and dimer are substantially different (Biswas & Pocker, 1979; Pocker & Biswas, 1980), an observation which made it possible to calculate the dimerization constant, K_{12}, at very high dilutions where a simple monomer ⇌ dimer equilibrium exists without any significant interference of higher associated states. These measurements have an advantage over equilibrium sedimentation studies, as the latter study is not possible at comparable dilutions with sufficient accuracy. We calculated for K_{12} the value of 7.5×10^5 M^{-1} (Table I), which is higher than, but comparable to, the values of 1.4×10^5 M^{-1} reported by Pekar & Frank (1972) at pH 7.0 and 2.22×10^5 M^{-1} reported by Goldman & Carpenter (1974) at pH 8.0 (Table II). The noted difference between our value and the last two values might be due to the difficulty of measuring the apparent weight-average molecular weight by equilibrium sedimentation at very low concentrations. By use of the value of 7.5×10^5 M^{-1} for K_{12}, values of K_{24} and K_{46} were varied continuously to compute $M_{w,app}$ and plotted against concentration. These plots were compared with $M_{w,app}$ vs. C plot of Goldman & Carpenter (1974). The best fit between theoretical and experimental plots was achieved with

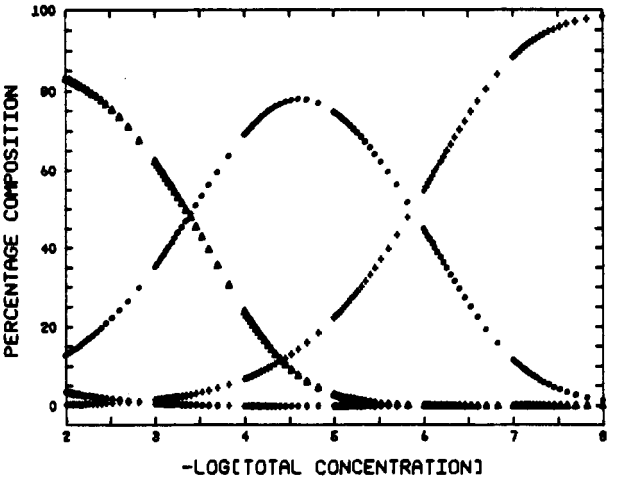


FIGURE 4: The theoretical distribution of various associated states of insulin as a function of the total concentration of insulin at pH 7.0 and 25 °C. Monomer (+); dimer (○); tetramer (△); hexamer (◇). This distribution was computed by using association constants reported in this paper (see Table II).

values of K_{24} and K_{46}, 5.0×10^3 M^{-1} and 45 M^{-1}, respectively (Table II; Figure 4). The excellent agreement between theoretical and experimental plots using these association constants is illustrated in Figure 2. We also examined the M–D–H model of insulin association, using $K_{12} = 7.5 \times 10^5$ M^{-1} and continuously varying K_{26}. However, using all possible values of K_{26}, we were unable to get a good fit between the computer-generated plot and the plot of Goldman & Carpenter (1974). Therefore, we conclude that the monomer ⇌ dimer ⇌ tetramer ⇌ hexamer model proposed by Jeffrey & Coates (1966) and by Goldman & Carpenter (1974) is a reasonable model for the association of Zn^{2+}-free insulin in solution.

Thermodynamics of Dimerization. The dimerization constant of insulin is relatively large (Table II), which indicates that a substantial change in standard free energy (ΔG° = −8.01 kcal mol^{-1}) is involved in insulin dimerization. Lord et al. (1973) calculated from UV spectroscopic studies a ΔG° of dimerization of −8.7 kcal mol^{-1}.

A high value of $\Delta G^{\circ}{}_{assoc}$ implies that there are several groups in the monomer which are involved in the dimerization process. Analysis of the X-ray crystallographic data of Blundell et al. (1971) gives a clear picture of the nature of the binding site in insulin and the side chains involved in insulin dimerization. These groups are extremely hydrophobic in nature, e.g., Phe, Tyr, and Val, and form a hydrophobic core in the monomer–monomer interface of the dimer. The interface resembles a drop of oil in a very polar and hydrophilic environment. The crystal structure indicates formation of four hydrogen bonds between the two monomers. However, H bonding between protein and water molecules is stronger than that between the two proteins (Tanford, 1970). Therefore, the hydrogen bonds are not contributing toward the stability of the dimer; on the contrary, these H bonds might cause small destabilization of the dimer. Clearly then, the major contribution is due to the hydrophobic bonding between the two monomers. We have attempted a semiquantitative estimation of the ΔG° of the insulin dimerization by using the thermodynamic parameters obtained from Némethy & Scheraga (1962) and from Nozaki & Tanford (1971). The values of δG° in eq 14 are given in Table I and II. The δG° values taken from Nozaki & Tanford (1971) are the free energies involved in transferring an individual side chain from water to 100% ethanol. Although a nonpolar side chain in 100% ethanol does not exactly represent the same side chain in a total nonpolar environment such

as an oil droplet, nevertheless, a comparison of the change in free energy of association calculated from the two different sets of values of δG°_i and δG°_{kj} gives us an opportunity to examine the reasonableness of this model.

A detailed analysis of the X-ray crystallographic data of Blundell et al. (1971) shows that the groups involved in each monomer are A_{19} Tyr, B_{12} Val, B_{16} Tyr, B_{24} Phe, B_{25} Phe, and B_{26} Tyr. The minimum contact distances between these groups are given in Table III.

In computing ΔG°_{assoc}, we calculated the intermonomer and intramonomer hydrophobic bonding arising from the proximity of the various side chains as seen in Table III. We assumed that the change in free energy due to conformational change is zero, which might lead to a small error. In order to calculate the intrinsic entropy change involving the degrees of freedom, we computed the changes in translational, rotational, and vibrational entropy involved in insulin dimerization by using the procedure of Steinberg & Scheraga (1963). In the present calculation, the molecular weight of monomeric insulin was taken as 5734, and the molecule was considered to be a solid sphere of radius 14.2 Å which includes a unimolecular layer of water molecules around the protein molecule. The calculated change in intrinsic entropy is −7.82 eu. This theoretical value of intrinsic entropy change is very close to the experimental value of Doty & Meyers (1953) and lies between the limits of experimental values 0 and −12 eu, depending on pH and buffer. The values for molecular weight and radius used in the derivation of the intrinsic entropy change are unquestionably accurate, so that the value of −7.82 eu has relatively small limits of uncertainty. By use of this value of ΔG°_{ent}, we calculated the net change in standard free energy for insulin dimerization. The computed value of ΔG°_{assoc} using the values of Némethy & Scheraga is −8.27 kcal mol^{-1} and using those of Nozaki and Tanford is −10.27 kcal mol^{-1} whereas the experimental value of ΔG°_{assoc} is −8.01 kcal mol^{-1}. The theoretical and experimental values are close, and the differences in the values fall within the limits of uncertainty. The difference of 2 kcal mol^{-1} between the two theoretical values arises clearly from the respective differences in δG° values. In the model described in this paper, we made two approximation: (i) we assigned a maximum value of 1.0 to all C_i and C_{kj} terms, and (ii) we ignored the contribution of conformational changes involved in insulin dimerization. Both of these approximations will lead to small errors in the value of ΔG°_{assoc}. Despite these approximations, the semiquantitative thermodynamic model gives reasonable results and furthermore establishes a good guide to other calculations involving δG° values of Némethy & Scheraga (1962). Further refinement is in progress.

The dimer-forming domain on the insulin surface is thus well characterized, and its importance in receptor binding has been elucidated by chemical and biochemical modifications, biological potency, and phylogenetic studies (Blundell et al., 1972; Peking Insulin Group, 1974; Pullen et al., 1976; Shanghai Insulin Group, 1978; Dodson et al., 1979). A similar entropic effect has been detected in receptor binding (DeMeyts & Waelbrock, 1977; Waelbrock et al., 1979), which proves that nonpolar residues and hydrophobic bonding are playing important roles in the receptor binding. Furthermore, enzymatic modifications of the B-chain C-terminal and biological potency studies of the modified insulin derivatives indicate that B_{24} Phe, B_{25} Phe, and B_{26} Tyr residues play an important role not only in dimerization but also in receptor binding and biological potency (unpublished results).

Studies in progress indicate that this model of protein association is fairly general and can be applied effectively with but minor modifications (such as addition of terms in eq 14 to account for conformational changes, ionic interactions, etc.) to rationalize the associative behavior of other proteins. Our studies indicate that an effective correlation can be established between theoretical association constants and experimental data on solution dynamics of several enzyme-modified insulin derivatives. This correlation can apparently be extended further to account for the biological potency of these derivatives (Y. Pocker and S. B. Biswas, unpublished results).

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Time-Resolved Fluorescence and Anisotropy Decay of the Tryptophan in Adrenocorticotropin-(1–24)†

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ABSTRACT: The direct time-resolved fluorescence anisotropy of the single tryptophan residue in the polypeptide hormone adrenocorticotropin-(1–24) (ACTH) and the fluorescence decay kinetics of this residue (Trp-9) are reported. Two rotational correlation times are observed. One, occurring on the subnanosecond time scale, reflects the rotation of the indole ring, and the other, which extends into the nanosecond range, is dominated by the complex motions of the polypeptide chain. The fluorescence lifetimes of the single tryptophan in glucagon (Trp-25) and the 23–26 glucagon peptide were also measured. In all cases the fluorescence kinetics were satisfied by a double-exponential decay law. The fluorescence lifetimes of several tryptophan and indole derivatives and two tryptophan dipeptides were examined in order to interpret the kinetics. In close agreement with the findings of Szabo and Rayner [Szabo, A. G., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563], the tryptophan zwitterion exhibits emission wavelength dependent double-exponential decay kinetics. At 320 nm $\tau_1 = 3.2$ ns and $\tau_2 = 0.8$ ns, with $\alpha_1 = 0.7$ and $\alpha_2 = 0.3$. Above 380 nm only the 3.2-ns component is observed. By contrast the neutral derivative *N*-acetyltryptophanamide has a single exponential decay of 3.0 ns. The multiexponential decay kinetics of the polypeptides are discussed in terms of flexibility of the polypeptide chain and neighboring side-chain interactions.

Polypeptide hormones in solution behave in many cases as fairly flexible chains. For example, before the recent developments in time-resolved fluorescence emission anisotropy [for review see Badea & Brand (1979)], suggestive but indirect evidence for dynamic motions in these molecules was obtained from a variety of physical techniques, including circular dichroism, optical rotatory dispersion, and steady-state fluorometry. In an elegant series of papers, Edelhoch and co-workers (Edelhoch & Lippoldt, 1969; Bernstein et al., 1969; Bornet & Edelhoch, 1971; Schneider & Edelhoch, 1972a,b) evaluated the conformational properties of model peptides and polypeptide hormones such as glucagon, adrenocorticotropin, parathyroid hormone, mellitin, and gastrin, and certain of their peptide fragments. The results confirm the notion that these are indeed flexible molecules, some of which can be induced to acquire secondary structure under a variety of circumstances. The polarization of the tryptophan fluorescence also suggested that the indole group was rotating at rates exceeding that expected if the hormones were to be considered as tumbling rigid spheres. When adrenocorticotropin and glucagon, each of which has a single tryptophan residue, were compared, it appeared that whereas the indole moiety was essentially unhindered in adrenocorticotropin, the results for glucagon suggested some steric restriction of the side chain. The NMR[1] spectrum of glucagon peptides is consistent with this notion (Bundi et al., 1976).

With the advent of time-resolved fluorescence anisotropy measurements, direct evidence was obtained for subnanosecond motions of tryptophans in several proteins (Munro et al., 1979). If it is possible to resolve the time-dependent motions in the polypeptides hormones, then a new avenue exists for exploring structure–function relationships. In this paper we present the results of one such test case, that of a biologically functional synthetic analogue of adrenocorticotropin, ACTH-(1–24) tetracosapeptide. Our results show that the single tryptophan residue rotates on the subnanosecond time scale, and furthermore this motion is the dominant factor responsible for the rapid depolarization of the tryptophan fluorescence. In addition, we find that the decay kinetics of the tryptophan fluorescence are complex and fit a double-exponential decay law which is essentially wavelength independent in neutral pH buffer. For comparison, we have measured the fluorescence lifetimes of the single tryptophan in glucagon and in its four-peptide fragment Val-Gln-Trp-Leu, which includes residues 23–26 of the 29 amino acid polypeptide chain [cf. Bromer et al. (1956)]. The decay data obtained with the polypeptides are compared with those obtained from simpler tryptophan systems under identical experimental conditions. The latter data, which include the decay kinetics of the tryptophan zwitterion, agree with the findings of Szabo &

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[1] Abbreviations used: ACTH, adrenocorticotropin hormone-(1–24); ADH, horse liver alcohol dehydrogenase; NATA, *N*-acetyltryptophanamide; NMR, nuclear magnetic resonance; ODMR, optically detected magnetic resonance.

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