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Kinetics and Thermodynamics of the α Helix $\rightleftarrows$ β Transconformation of Poly(L-lysine) and L-Leucine Copolymers. A Compensation Phenomenon†

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ABSTRACT: The kinetics of the transition α helix to β form of poly(L-lysine) at pH 11.4 in 0.05 M KF solution has been studied, and for two copolymers of L-lysine and L-leucine containing 0.065 and 0.11 mol fraction of leucine. These studies were performed at a concentration such that the β form produced was only the intramolecular structure. The activation parameters, $\Delta H_{\alpha\rightarrow\beta}^{\ddagger}$, $\Delta S_{\alpha\rightarrow\beta}^{\ddagger}$, and $\Delta G_{\alpha\rightarrow\beta}^{\ddagger}$, were obtained from the temperature dependence of the first-order rate constant for the transition. As the leucine content of the polymers increases, the values of $\Delta H_{\alpha\rightarrow\beta}^{\ddagger}$ and $\Delta S_{\alpha\rightarrow\beta}^{\ddagger}$ become less positive as follows: for $\Delta H_{\alpha\rightarrow\beta}^{\ddagger}$, 64.3, 54.7, and 37.6 kcal mol^{-1}; and for $\Delta S_{\alpha\rightarrow\beta}^{\ddagger}$, 130, 100, and 45 eu. This is interpreted as a disruption of hydrophobic interactions and consequent interaction of the side chains with solvent. The importance of hydrophobic interactions on proceeding to the transition state is emphasized by the linear compensation of $\Delta H_{\alpha\rightarrow\beta}^{\ddagger}$ and $\Delta S_{\alpha\rightarrow\beta}^{\ddagger}$, with a compensation temperature of 302.5°K. The compensation is interpreted as being due to the volume change accompanying solvation of hydrophobic side chains producing an opposing volume change in the environmental solvent, the solvent volume change being a "hydrogen-bond making process" of the two-state water theory. The derived ratio of ΔH:ΔS for such a process is 300°K [Lumry, R., and Rajender, S. (1970), *Biopolymers 9*, 1125], which is in good agreement with the data herein. The reverse transition for each polymer from the intramolecular β form to the helix was studied at 277°K in 0.01 M boric acid, pH 10.6; the values of $\Delta G_{\beta\rightarrow\alpha}^{\ddagger}$ were calculated and the values of $\Delta G_{\alpha\rightleftarrows\beta}^{\ddagger}$ (277°K) were computed. A molecular description of the transition and transition state is postulated from the experimental data and a comparison of the stabilities of these β structures for the series of polymers was obtained.

As the number of X-ray studies of proteins and enzymes (giving complete three-dimensional structures) increases, it is becoming obvious that the α helix can no longer be called the most important secondary structure present in such biological macromolecules. Instead, it is apparent that nonperiodic and β forms occur more frequently than the helical form. Very little is known about the nonperiodic form which is probably stabilized by forces involving interactions between residues which are distant from each other on the peptide chain and are brought into juxtaposition by folding. The structures showing periodicity are much easier to analyze in terms of molecular forces and physical properties; the greatest body of knowledge of such structures is undoubtedly that of the α-helical form. The other periodic structure, the β form, has been much less intensively studied mainly due to experimental problems, *e.g.*, solubility. The problems of studying the β structure in proteins are innumerable. Thus, as with the α helix, resort has been made to model compounds.

Low molecular weight poly(α-amino acids), such as poly(γ-benzyl-L-glutamate) (Fasman, 1967; Imae and Ikeda, 1972; Ikeda and Imae, 1972) and isoleucine oligomers (Blout and Shechter, 1963; Goodman *et al.*, 1971; Widmer and Lorenzi, 1971), assume a β form as do homopolymers of L-serine (Tooney and Fasman, 1968; Quadrifoglio and Urry, 1968), *S*-carboxymethyl-L-cysteine (Ikeda and Fasman, 1967; Makino and Sugai, 1970; Makino and Noguchi, 1971), *S*-carboxyethyl-L-cysteine (Maeda and Ikeda, 1971a,b), and L-lysine (Davidson *et al.*, 1966; Sarkar and Doty, 1966; Townend *et al.*, 1966; Fasman and Potter, 1967; Davidson and Fasman, 1967; Li and Spector, 1969; Wooley and Holzwarth, 1970; Pederson *et al.*, 1971). The β form has also been reported for

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polymers of L-histidine (Myer and Barnard, 1971) and L-tyrosine (Conio *et al.*, 1971) under certain conditions. Poly(L-lysine) has received considerable attention as this polymer can be obtained in the α helical, nonperiodic, or β form depending on the environmental conditions. The β structure is not in itself a pure conformational species as it is possible to fulfill the molecular geometric requirements with three different forms. If the β form is composed of the association of different peptide chains, then the chains have either the same (parallel) or alternating (antiparallel) direction (Pauling and Corey, 1951); however if the association is of the same polypeptide chain then a cross-β form is produced which necessarily must be antiparallel (Astbury *et al.*, 1935). The intramolecular β form has been observed in many globular proteins, *e.g.*, lysozyme (Blake *et al.*, 1965), papain (Drenth *et al.*, 1968), and lactate dehydrogenase (Rossman *et al.*, 1971). This cross-β conformation has been found in many denatured proteins (Senti *et al.*, 1943) and in certain synthetic polypeptides of low molecular weight (Bradbury *et al.*, 1960). The most perfect example is found in the silk from egg stalks of the green lace wing fly (Parker and Rudall, 1957).

Clearly if a polymer assumes the β conformation the possibility of intra- or intermolecular β structure will be concentration dependent. This in fact was found by Wooley and Holzwarth (1970) to be the case for poly(L-lysine), the intramolecular form being obtained at low polymer concentration. These workers studied the transition from α helix to β form as a function of concentration and found that the kinetics were first order in α-helix concentration when the intramolecular form was obtained. The β form poly(L-lysine) assumed at higher concentration is thought to be an intermolecular antiparallel structure (Davidson and Fasman, 1967). The transformation of poly(L-lysine) from the α helix to the β form is induced thermally (Rosenheck and Doty, 1961; Applequist and Doty, 1958, 1962; Sarkar and Doty, 1966; Davidson and Fasman, 1967). Davidson and Fasman (1967) also found that the β form of poly(L-lysine) was able to revert slowly back to the α-helical form at 277°K.

The work described herein is a study of the interconversion of the α-helical and intramolecular β structure of poly(L-lysine) and copolymers of L-lysine and L-leucine. From the kinetic behavior of the $\alpha \rightarrow \beta$ transition, as a function of temperature, the activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ are evaluated for each polymer;[1] from these parameters it is deduced that passage through the transition state is mediated by hydrophobic interactions. Additional information on the participation of hydrophobic interactions in the transition state is obtained from the compensation behavior of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Such compensation phenomena have been used to intimate the participation of solvent in the transition state, as the phenomenon is thought to reflect a common property of the solvent (Lumry and Rajender, 1970). This behavior has been described thermodynamically, as the main physiologically important property of proteins in both equilibria and rate processes (Lumry and Eyring, 1954). $\Delta G^{\ddagger}$ values are obtained for each polymer for the transition from $\beta \rightarrow \alpha$ at 277°K. Thus, for the first time, quantitative measures of the relative stability of the β forms of a series of polymers in aqueous media are obtained, along with deductions as to the molecular forces governing the transitions observed.

Materials and Methods

Poly(L-lysine) was synthesized as previously described (Fasman *et al.*, 1961).

L-Leucine N-carboxyanhydride was prepared as described by Fasman *et al.* (1964).

Copolymers of L-Lysine and L-Leucine. A typical polymerization for a 90:10 L-lysine–L-leucine copolymer is as follows: ϵ-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride (0.700 g; 2.285 mmol) and L-leucine *N*-carboxyanhydride (0.406 g; 0.2583 mmol) were dissolved in freshly distilled dioxane (74 ml).[2] The solution was stirred and 0.397 M sodium methoxide [0.0672 ml; A:I (anhydride:initiator) ratio = 100] was added with stirring and then the solution was allowed to polymerize by standing overnight. Hydrogen chloride gas was bubbled through the solution for 15 min followed by hydrogen bromide gas for 30 min. The solution was stirred for 90 min and then nitrogen gas was bubbled through the solution for 90 min to remove both HCl and HBr. All the above procedures were carried out with careful exclusion of moisture. The solvent was evaporated on a rotary evaporator and the resultant solid was washed twice with ether and dissolved in water. The pH of the aqueous solution was adjusted to 5 with 1 M sodium hydroxide and the solution was dialyzed against 0.01 M hydrochloric acid. The final solution was filtered through a Millipore filter (5 μ pore; Millipore Corp., Bedford, Mass.) to remove any undissolved material, lyophilized, and dried *in vacuo* for 2 hr at 80°; yield 0.205 g (50.5%), $[\eta]^{1\text{ M NaCl}}_{\text{pH }4.2}$ = 1.59. Table I shows the molar ratio of the *N*-carboxyanhydrides and the A:I ratio used in the polymerization reaction and the composition of the various polymers obtained. The compositions were obtained by amino acid analysis on a Beckman Model 120C analyzer by the method of Spackman *et al.* (1958) after complete hydrolysis in 6 M HCl for 24 hr at 110° *in vacuo*. The intrinsic viscosities are also shown in Table I along with the molecular weight estimates calculated from the calibration curve of Applequist (1959) for poly(L-lysine).

Viscometry. Intrinsic viscosities were determined using Ubbelohde viscometers at a temperature of 25 ± 0.1°.

Circular dichroism (CD) measurements were made on a Cary 60 spectropolarimeter fitted with Model 6001 circular dichroism attachment. The slit width was programmed to a 15-Å half-bandwidth; ambient temperature of the instrument was 296°K. The circular dichroism system was calibrated with *d*-camphorsulfonic acid using the method of Adler *et al.* (1972). The Cary 60 was always operated at scale expansion 0.04° and time constant 0.3 sec with a 1-cm path-length cell.

pH measurements were performed using a Radiometer 25 pH meter with a Radiometer Type GK 2302G combination glass electrode. The electrode was calibrated using standard buffers from Fisher Scientific Co.

Kinetics of the $\alpha \rightarrow \beta$ transition were observed as follows. A stock solution of polymer in 0.05 M KF was diluted to the required concentration and the solution adjusted to pH 11.40 and filtered, through a Millipore filter (5 μ pore, Millipore Corp., Bedford, Mass.), using a 10-ml B-D Multifit syringe (Becton, Dickinson Co., Rutherford, N. J.) into a 1-cm jacketed CD cell (Optical Cell Co., Beltsville, Md.). The CD

[1] The thermodynamic relations and symbols follow the convention that the superscript ‡ indicates transition state and the subscript $\alpha \rightarrow \beta$ or $\beta \rightarrow \alpha$ indicates the direction of the transition; ΔH, ΔG, and ΔS are the changes in enthalpy, Gibbs free energy, and entropy, respectively. The abbreviations for amino acids and polymers conform to the tentative rules of the IUPAC–IUB Commission on Biochemical Nomenclature, as published in *Biochem. J. 102*, 23 (1967), and *J. Biol. Chem. 247*, 323 (1972).

[2] Purified by the Feiser Method (Fieser, 1941).

TABLE I: L-Lysine and L-Leucine Copolymers.[a]

Polymer No.	Lys:Leu Anhydride Ratio	A:I[b]	Lys:Leu Ratio from Amino Acid Anal.[c]	$[\eta]^{1M\ NaCl}_{pH\ 4}$	Mol Wt[d]	DP
I (GF-19-39-27)	100:0	200	100:0	1.82	133,000	808
II (GF-18-126-14)	95:5	100	93.5:6.5	1.79	129,000	799
III (GF-18-124-16)	90:10	100	89:11	1.59	114,000	717

[a] Polymerization conditions, lysine:leucine ratios and molecular weights. [b] $NaOCH_3$; used as initiator: A:I, anhydride to initiator ratio. [c] Method of Spackman *et al.* (1958). [d] Estimated from the calibration curve of Applequist (1959).

cell was connected to 2 Haake circulation baths (Model FJ) in parallel so that the temperature of the cell and contents could be changed rapidly. The temperature of the sample was determined immediately after the exit of the cell jacket using a Type 423 thermistor probe and a YSI telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The ellipticity of the sample at 222 nm was followed as a function of time at the desired temperature.

Kinetics of the $\beta \rightarrow \alpha$ transition were observed as follows. A stock polymer solution in 0.01 M boric acid was diluted to give the required polymer concentration and the pH adjusted as necessary to an accuracy of 0.01 pH unit. The solution was filtered through a Millipore filter (5 μ pore) and placed in a thermostatted heating bath at a temperature necessary to convert the polymer to the β form. After conversion to the β form, the solution was cooled to 277°K and placed over sodium hydroxide flakes in a vacuum desiccator, precooled to 277°K. The kinetics of the conversion to the α form were followed at 277°K by observing the CD of aliquots of the solution at 277°K taken at different times through the conversion. In all cases the aliquot was warmed to 296°K before the CD was run to eliminate errors due to temperature variability; it was assumed that warming to 296°K effectively froze the kinetic process relative to the time scale of a CD run.

Results

Transition from α Helix $\rightarrow$ β Form. This transition was observed with each of the polymers as a function of temperature and at a concentration low enough to ensure the formation of only the *intramolecular* β structure. If the intramolecular β form is obtained, then the kinetic process will be first order. The concentration necessary to obtain first-order kinetics was established using the fractional lifetime method (Frost and Pearson, 1961) in a manner analogous to Wooley and Holzwarth (1970). The concentrations used are listed in Table III. The kinetics of the $\alpha \rightarrow \beta$ transition were followed using circular dichroism measurements by observing the ellipticity at 222 nm, $[\theta]_{222}$, as a function of time at any fixed temperature; the Cary 60 was used at maximum pen response to ensure reliability of the data at temperatures where the transition is rapid. The initial rapid change in $[\theta]_{222}$ at the beginning of the transition was not treated as part of the $\alpha \rightarrow \beta$ kinetic process but was assumed to reflect a rapid equilibration of the polymer solution after the desired temperature of the water jacket was reached (Davidson and Fasman, 1967). Wooley and Holzwarth (1970) assign this change to a transition from 100% helix to a mixture of helix and irregular structure.

The change of the fraction of β form present, as a function of temperature and time, is shown for polymers I, II, and III in Figures 1, 2, and 3, respectively. The fraction β, f_β, is obtained from the relationship

$$f_\beta = \frac{[\theta]_{222}^{\alpha} - [\theta]_{222}^{t}}{[\theta]_{222}^{\alpha} - [\theta]_{222}^{\beta}} \quad (1)$$

where $[\theta]_{222}^{t}$ is the ellipticity at time t, $[\theta]_{222}^{\alpha}$ and $[\theta]_{222}^{\beta}$ are the ellipticity values at zero time and infinity, respectively. The process is assumed to be a two-state process of the form: $\alpha \rightleftarrows \beta$. No evidence was seen, in the CD spectra, to indicate the participation of a third component such as the nonperiodic structure. The kinetic data were analyzed to obtain the first-order rate constant, k, in the standard manner (Glasstone *et al.*, 1941), from the relationship

$$\ln (1 - f_\beta) = -kt \quad (2)$$

The variation of k with temperature for each polymer is shown in Table II. k can be expressed by the following formula from the transition-state theory (Glasstone *et al.*, 1941)

$$k = \frac{k_B T}{h} e^{-\Delta H^{\pm}/RT} e^{\Delta S^{\pm}/R} \quad (3)$$

where k_B is the Boltzmann constant, h is Planck's constant, R is the gas constant, T is the absolute temperature and $\Delta H^{\pm}$ and

TABLE II: Variation of the First-Order Rate Constant, k, with Temperature (°K), pH 11.40 for the $\alpha \rightarrow \beta$ Transition, of Poly(L-Lys), Poly[$Lys^{93.5}Leu^{6.5}$] and Poly[$Lys^{89}Leu^{11}$].

Poly-L-Lys		Poly[$Lys^{93.5}Leu^{6.5}$]		Poly[$Lys^{89}Leu^{11}$]	
T (°K)	$k \times 10^3$ (sec^{-1})	T (°K)	$k \times 10^3$ (sec^{-1})	T (°K)	$k \times 10^3$ (sec^{-1})
317.0	0.663	318.0	1.12	326.5	0.693
317.9	1.26	320.0	1.19	327.7	0.887
318.2	1.34	322.0	3.92	330.4	1.46
319.8	2.20	324.2	6.425	331.0	1.68
320.1	2.09	326.0	11.56	332.5	2.98
321.0	4.59	327.9	18.48	335.5	4.24
322.5	7.45	329.8	21.19	338.0	8.46
322.5	5.13			339.8	5.76
323.5	8.73			340.5	8.12
326.0	13.89			343.0	11.05
328.0	21.37				

TABLE III: Enthalpy, Entropy, and Free Energy of Activation for the α Helix $\rightarrow$ β Form Transition and Ellipticity of β form at 217-nm Maximum, $[\theta]_{217}$, at pH 10.6.

Polymer No.	Lys:Leu Amino Acid Ratio	$[\theta]_{217}$[a] ((deg cm^2) dmol^{-1})	$\Delta H_{\alpha\rightarrow\beta}^{\mp}$ (kcal mol^{-1})	$\Delta S_{\alpha\rightarrow\beta}^{\mp}$ (eu)	$\Delta G_{\alpha\rightarrow\beta}^{\mp}$ (298°K) (kcal mol^{-1})	Concn (g $\times$ 10^3/100 ml)
I	100:0	$-18,000 \pm 1800$	64.3	130.5	25.4	2.2
II	93.5:6.5	$-22,000 \pm 2200$	54.7	100	24.9	2.5
III	89:11	$-23,000 \pm 2300$	37.6	42.5	25.0	0.75

[a] Ellipticity at maximum for β conformation.

$\Delta S^{\mp}$ are the enthalpy and entropy of activation, respectively. From a plot of log k *vs.* $1/T$, $\Delta H^{\mp}$ and $\Delta S^{\mp}$ can be obtained from the slope and intercept, respectively, of the resultant straight line. Figures 4, 5, and 6 show the log k *vs.* $1/T$ plots for polymers 1, 2, and 3, respectively; the data were fitted using a linear regression analysis. The values of $\Delta H_{\alpha\rightarrow\beta}^{\mp}$, $\Delta S_{\alpha\rightarrow\beta}^{\mp}$, and thus $\Delta G_{\alpha\rightarrow\beta}^{\mp}$ obtained in this manner are tabulated in Table III. The standard state is unity in all cases. It is clear from these data that compensatory behavior is being observed whereby the extrathermodynamic relationship

$$\Delta H^{\mp} = \alpha + T_c \Delta S^{\mp} \quad (4)$$

is obeyed (Leffler and Grunwald, 1963). This is seen more clearly in Figure 7 in which $\Delta H^{\mp}$ is plotted against $\Delta S^{\mp}$; the data were fitted by a linear regression analysis and the straight line obtained has a correlation coefficient of better than 0.99 with the data points. The slope of the line, T_c, is called the compensation temperature, and in this case is 302.5°K.

Transition from the $\beta \rightarrow \alpha$ Helix. This reverse transition had been observed previously by Davidson and Fasman (1967) but was not studied in any detail. All three polymers undergo a transition from the β form $\rightarrow$ α helix at 277°K. The kinetics of the transition were followed at the same concentration as in the $\alpha \rightarrow \beta$ transition and at pH 10.60; it was found that the conversion became slower as the pH was raised and at pH 11.4, the transition was too slow to be observed over a period of 48 hr. Therefore the pH was lowered to a point where the rate was sufficient to be adequately measured. It was necessary to buffer the solution with 0.01 M boric acid, as in the absence of buffer the pH drifted considerably over the time period required for observation of the kinetics. A graph of log $(1 - f_\beta)$ *vs.* time is shown in Figure 8, and the first-order rate constant is obtained from the slope. The rate constant for each polymer for the $\beta \rightarrow \alpha$ transition at 227°K is seen in Table IV, and also shown is $\Delta G_{\beta\rightarrow\alpha}^{\mp}$ (277°K) which is the free energy of activation for the $\beta \rightarrow \alpha$ transition at 277°K, given by

$$\Delta G^{\mp} = -RT \ln K^{\mp} \quad (5)$$

where $K^{\mp} = kh/k_B T$ (Glasstone *et al.*, 1941); the symbols are defined above.

Discussion

In the experiments described in this study the β form obtained was always the *intramolecular* structure, as is clear from the first-order nature of the transition; this is important because if there is partial intermolecular β structure present, the kinetics of the $\alpha \rightarrow \beta$ transition will not be first order but of mixed order, thus increasing the complexity of the kinetic

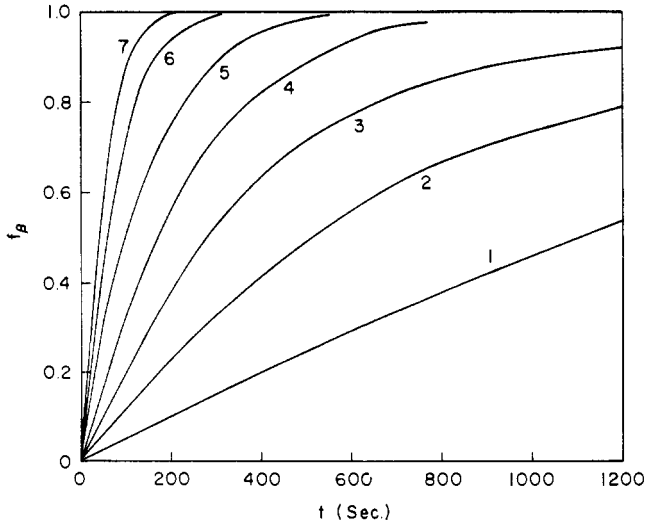

FIGURE 1: Graph of the fraction of β form, f_β, as a function of time, t, in seconds for the α helix $\rightarrow$ β transition of poly(L-lysine) at the following temperatures (°K): 1, 317°; 2, 318.2°; 3, 319.8°; 4, 321°; 5, 322.5°; 6, 326°; 7, 328°. Polymer concentration, 2.2 mg/100 ml; solvent, 0.05 M KF; cell path length, 1 cm, pH 11.40.

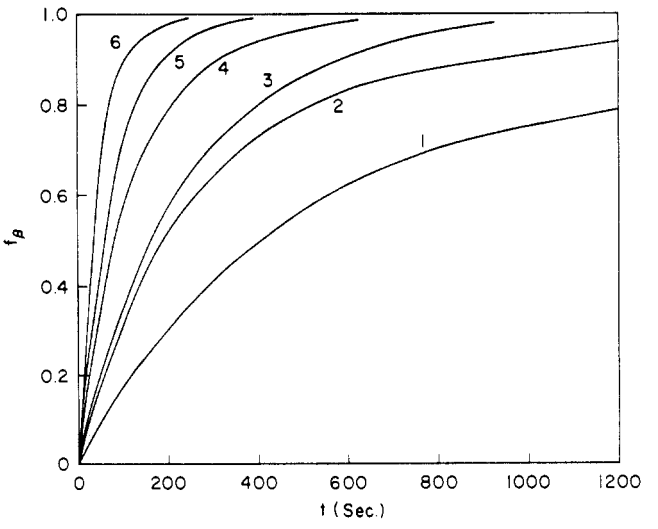

FIGURE 2: Graph of the fraction of β form, f_β, as a function of time, t, in seconds for the $\alpha \rightarrow \beta$ transition of poly[$Lys^{93.5}$ $Leu^{6.5}$] at the following temperatures (°K): 1, 318°; 2, 320°; 3, 322°; 4, 324.2°; 5, 326°; 6, 329.8°. Polymer concentration, 2.5 mg/100 ml; solvent 0.05 M KF; cell path length, 1 cm, pH 11.40.

TABLE IV: The Degree of Ionization, α, Rate Constant, $k_{\beta\rightarrow\alpha}$, and Free Energy of Activation, $\Delta G_{\beta\rightarrow\alpha}{}^{\ddagger}$, for the $\beta \rightarrow \alpha$ Transition Compared to the Free Energy of Activation, $\Delta G_{\alpha\rightarrow\beta}{}^{\ddagger}$, for the $\alpha \rightarrow \beta$ Transition at 277°K, and the Computed Free Energy, $\Delta G_{\alpha\rightleftarrows\beta}$, for the $\alpha \rightleftarrows \beta$ Equilibrium.

Polymer No.	Lys:Leu Ratio	α[a] (pH 10.6, 298°K)	Rate Constant $k_{\beta\rightarrow\alpha} \times 10^4$	$\Delta G_{\beta\rightarrow\alpha}{}^{\ddagger}$ (277°K) (kcal mol^{-1})	$\Delta G_{\alpha\rightarrow\beta}{}^{\ddagger}$ (277°K) (kcal mol^{-1})	$\Delta G_{\beta\rightleftarrows\alpha}$ (277°K) (kcal mol^{-1})
I	100:0	0.840	1.42	21.0	28.2	−7.2
II	93.5:6.5	0.855	0.701	21.4	27.0	−5.6
III	89:11	0.852	0.419	21.7	25.9	−4.2

[a] Snell and Fasman (1972).

analysis. In the kinetic analysis, it was assumed that the transition involved is a two-state process, *i.e.*, $\alpha \rightleftarrows \beta$. The fact that the transition involved could be satisfied by first-order kinetics indicates that α is either the helical form only or a constant mixture of α-helical and nonperiodic form (i), *i.e.*, $(\alpha \rightleftarrows \text{i}) \rightarrow \beta$. This condition appeared to be satisfied by analysis of the CD spectrum obtained. Although no evidence from the CD data was obtained to indicate the participation of the irregular form in the kinetic process, the participation of a small amount of random structure as a possible intermediate cannot be overlooked. The maximal ellipticity values for the α-helical forms were as reported by Snell and Fasman (1972); the extremum ellipticity values occur at 217 nm for the β form of the three polymers at pH 10.60 in 0.05 M KF solution. The values of $[\theta]_{217}$ are listed in Table III. The errors of measurement are large due to the low polymer concentrations necessary to ensure intramolecular β structure only. The data indicate that as the leucine content of the polymers increases, the value of $[\theta]_{217}$ becomes more negative. This could be due to increased rigidity of the β form as the leucine content increases; however, the importance of the differences is difficult to assess as the errors are only slightly less than the differences observed. The thermodynamic parameters for the activation process, $\Delta H_{\alpha\rightarrow\beta}{}^{\ddagger}$, $\Delta S_{\alpha\rightarrow\beta}{}^{\ddagger}$, and $\Delta G_{\alpha\rightarrow\beta}{}^{\ddagger}$, yield valuable information about the nature and course of the transition.

The fact that $\Delta S_{\alpha\rightarrow\beta}{}^{\ddagger}$ is large and positive indicates the degree of unfolding of the polypeptide chain is significant in the transition state relative to the ground state (helical form). $\Delta S_{\alpha\rightarrow\beta}{}^{\ddagger}$ decreases in magnitude as the leucine content of the polymers increases, suggesting the transition state, consisting of polypeptide chain and environmental solvent, becomes more ordered as the leucine content increases. Simplistically, the entropic contribution to the free energy of activation can be considered to be made up of two opposing factors: the entropy change due to the breaking of hydrogen bonds and unfolding of the polypeptide chain, and the other entropic contribution due to the exposure of hydrophobic residues to an aqueous environment. The latter process is necessarily negative due to the solvent ordering process around the hydrophobic groups (Kauzmann, 1959). The former entropic change would be expected to be positive (Brandts, 1969); therefore it seems reasonable to deduce that if $\Delta S_{\alpha\rightarrow\beta}{}^{\ddagger}$ decreased as the number of strongly hydrophobic residues increased (leucine residues), then the transition state must involve exposure of the hydro-

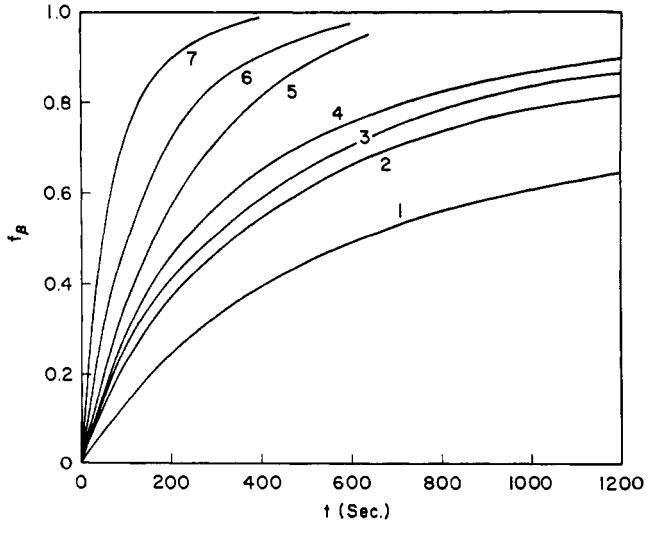

FIGURE 3: Graph of fraction of β form, f_β, as a function of time, t, in seconds for the $\alpha \rightarrow \beta$ transition of poly(Lys^{89}Leu^{11}] at the following temperatures (°K): 1, 326.5°; 2, 327.7°; 3, 331°, 4, 332.5°; 5, 335.5°; 6, 339.8°; 7, 343°. Polymer concentration, 0.75 mg/100 ml; solvent, 0.05 M KF; cell path length, 1 cm, pH 11.40.

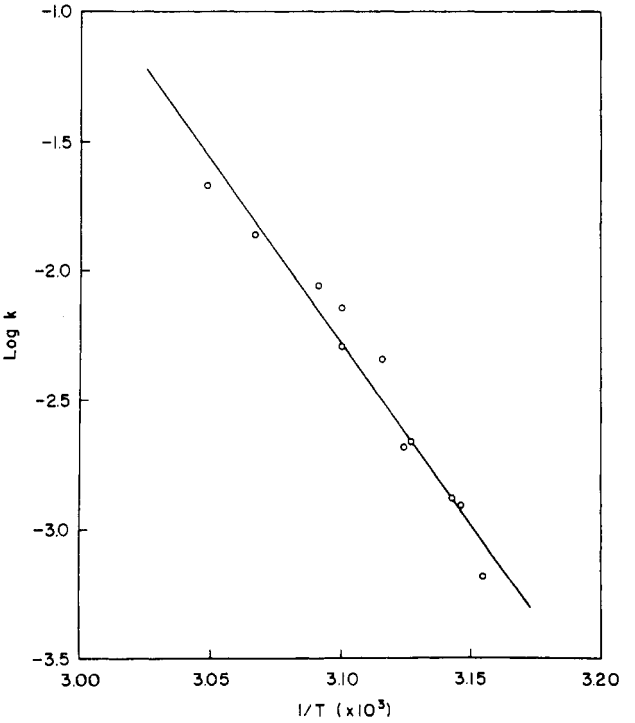

FIGURE 4: Van't Hoff plot of the logarithm of the first-order rate constant, log k, as a function of the reciprocal of the absolute temperature, $1/T$, for poly(L-lysine).

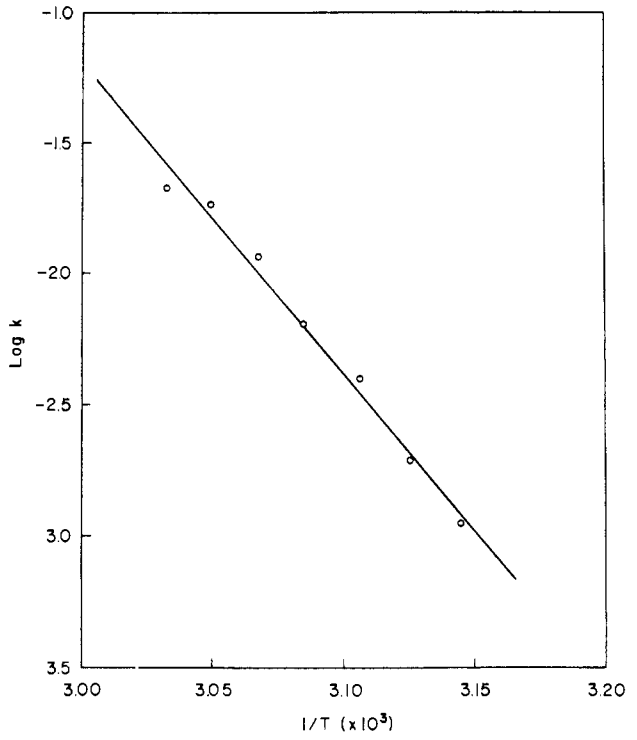

FIGURE 5: Van't Hoff plot of the logarithm of the first-order rate constant, log k, as a function of the reciprocal of the absolute temperature, $1/T$, for poly[Lys$^{93.5}$Leu$^{6.5}$].

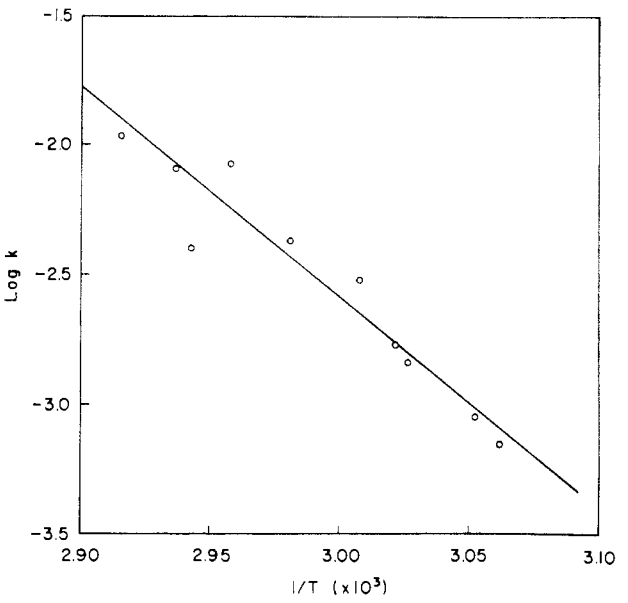

FIGURE 6: Van't Hoff plot of the logarithm of the first-order rate constant, log k, as a function of the reciprocal of the absolute temperature, $1/T$, for poly[Lys^{89}Leu^{11}].

phobic residues to the solvent. From the magnitude of $\Delta S_{\alpha\rightarrow\beta}{}^{\mp}$ alone it is impossible to assess the relative contributions of hydrophobic and conformational entropic components to the activation process; thus although $\Delta S_{\alpha\rightarrow\beta}{}^{\mp}$ is large and positive it could be made up of a very large conformational term and a significant negative hydrophobic term.

The magnitude and variation of $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ are necessarily less important than $\Delta S_{\alpha\rightarrow\beta}{}^{\mp}$ in discussing the hydrophobic bonding contribution, as the stabilization (or destabilization) of hydrophobic interactions is entropically controlled (Kauzmann, 1959; Némethy and Scheraga, 1962a,b). Nevertheless, from studies of model compounds it is possible to predict the behavior of ΔH on formation of a hydrophobic interaction. The treatment of Némethy and Scheraga (1962a,b), which is based on such model studies, predicts that exposure of two hydrophobic groups involved in hydrophobic interactions, to aqueous solvent, causes a decrease in the enthalpy of between 0.3 and 1.8 kcal mol^{-1}. These workers suggest an approximate value for ΔH of -1.4 kcal mol^{-1} for such a process between a lysine and a leucine residue. In the absence of a value for this process between two lysine groups, comparison of the values obtained for $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$, shown in Table III, with theory cannot be made. Nevertheless, it would be expected that the ΔH for complete solvation of the groups involved in hydrophobic interaction would be more negative for a lysine–leucine than a lysine–lysine interaction, and greater still for a leucine–leucine interaction. The value of $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ (Table III) becomes less positive as the leucine content of the polymers increases. This can be rationalized as follows. If the hydrophobic interactions are broken, exposing the side chains to solvent in the transition state, then as the leucine content increases the number of lysine–leucine interactions will increase and thus the value of $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ would be expected to decrease. Naturally the absolute values of $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ contain unknown contributions from the unfolding of the polypeptide chain, $\Delta H_{\text{conf}}{}^{\mp}$, the breaking of internal hydrogen bonds, $\Delta H_{\text{H}}{}^{\mp}$, and the hydrophobic interactions discussed above, $\Delta H_{\text{H}\phi}{}^{\mp}$, *i.e.*,

$$\Delta H_{\alpha\rightarrow\beta}{}^{\mp} = \Delta H_{\text{conf}}{}^{\mp} + \Delta H_{\text{H}}{}^{\mp} + \Delta H_{\text{H}\phi}{}^{\mp} \qquad (6)$$

$\Delta H_{\text{conf}}{}^{\mp}$ is not well understood but can be assumed to be positive (Tanford, 1970); values for $\Delta H_{\text{H}}{}^{\mp}$ reported in the literature vary considerably in magnitude but are positive (Tanford, 1970); and $\Delta H_{\text{H}\phi}{}^{\mp}$ is negative (Némethy and Scheraga, 1962a,b). The values of $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ in Table III undoubtedly contain a large contribution from $\Delta H_{\text{conf}}{}^{\mp}$, with a negative contribution from $\Delta H_{\text{H}\phi}{}^{\mp}$ and an unknown positive contribution from $\Delta H_{\text{H}}{}^{\mp}$.

Despite the lack of knowledge of magnitude of each contribution to $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ and $\Delta S_{\alpha\rightarrow\beta}{}^{\mp}$, the significant decrease in both $\Delta H_{\alpha\rightarrow\beta}{}^{\mp}$ and $\Delta S_{\alpha\rightarrow\beta}{}^{\mp}$ as the leucine content of the copolymers increases clearly indicates the sizeable contribution from hydrophobic interactions in proceeding to the transition state. The importance of hydrophobic interactions in the poly(L-lysine) matrix cannot be estimated from these data alone, but previous workers have suggested that these molecular forces are important (Némethy and Scheraga, 1962a; Scheraga *et al.*, 1962; Davidson and Fasman, 1967; Noguchi and Yang, 1971).

The fact that the thermodynamic data for the $\alpha \rightarrow \beta$ transition show enthalpy–entropy compensation yields additional information on the nature of the transition state. Such compensation phenomena have been observed in widely differing biological systems (Lumry and Rajender, 1970) as well as in numberable chemical and physical processes (Leffler and Grunwald, 1963). The detailed origin of such extrathermodynamic phenomena is, as yet, unknown but is considered to be a consequence of the properties of liquid water as a solvent regardless of the solute, due to temperature-independent heat capacity changes and/or shifts in concentration of the two phenomenologically significant species of water (Lumry and Rajender, 1970). The compensation observed in this work is produced when a given chemical structure (poly(L-lysine)) is

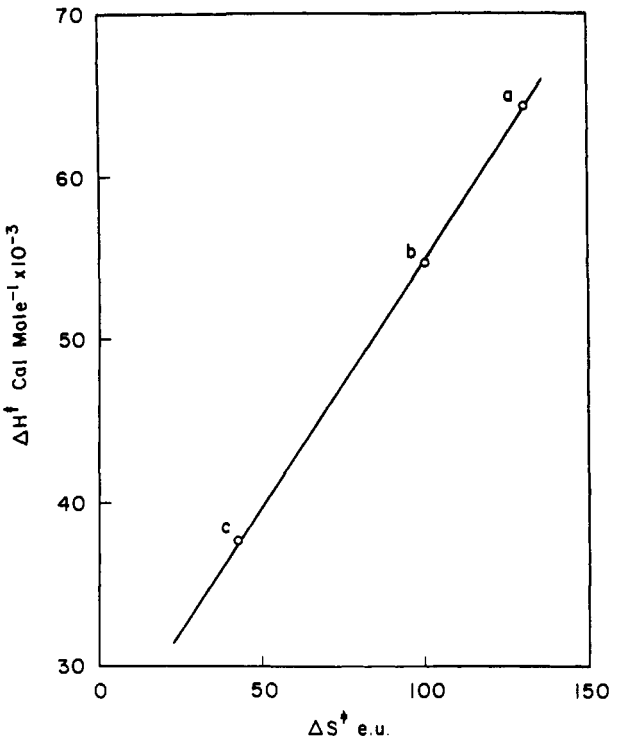

FIGURE 7: Compensation plot of $\Delta H^{\pm}$ as a function of $\Delta S^{\pm}$. The points are labeled as follows: (a) poly(L-lysine), (b) poly[Lys$^{93.5}$-Leu$^{6.5}$], and (c) poly[Lys^{89}Leu^{11}].

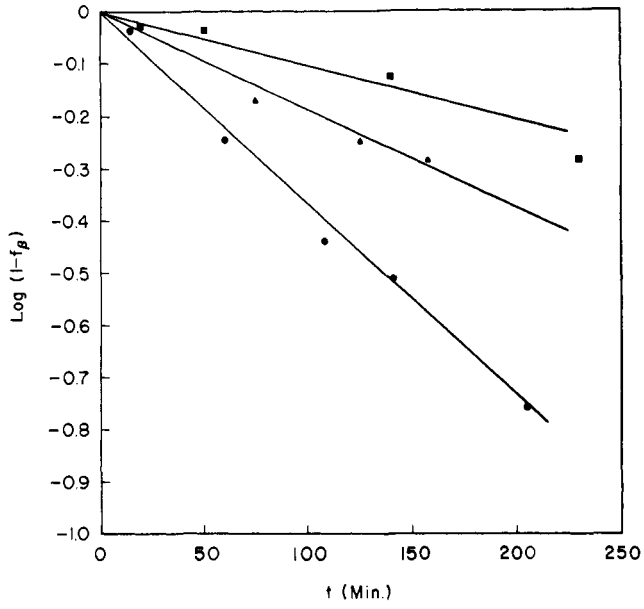

FIGURE 8: First-order rate plot of log $(1 - f_\beta)$ as a function of time, t, in minutes for poly(L-lysine) (●), poly[Lys$^{93.5}$Leu$^{6.5}$] (▲), and poly[Lys^{89}Leu^{11}] (■).

varied to give a homologous series of components for a kinetic process. The fact that compensation is observed indicates the similarity of the processes involved in proceeding to the transition state for all the polymers, and is a direct consequence of the solvent. Lumry and Rajender (1970), in a comprehensive survey of the literature, found in aqueous systems that T_c, the compensation temperature, lies between 250 and 320°K, with the greatest frequency between 270 and 290°K. The compensation plot shown in Figure 7 gives a T_c value of 302.5°K, which falls well within the former range. The direct effect of the compensation phenomenon is to ensure little, if any, variation of $\Delta G_{\alpha\rightarrow\beta}{}^{\pm}$ with the chemical composition of the polymer, as can be seen in Table III. It seems a reasonable deduction that the common solvent property which gives rise to the compensation effect is involved with the hydrophobic interaction processes associated with going from the ground state to the transition state. If exposure of hydrophobic side chains to an aqueous environment is involved in the transition state then there should be a decrease in polymer volume. Kauzmann (1959) estimated that the protein partial molal volume should decrease by about 20 ml, at room temperature, for each aliphatic side chain which is transferred from a nonpolar environment to water. The measured volume increment of +3.8 ml of water for an amino acid residue in poly(L-lysine) going from α helix to the β form in 0.2 M NaBr has been reported by Noguchi and Yang (1971). This of course does not reflect the transition state but only the difference between the starting (α) and final (β) conformation. Nevertheless, these data do suggest that the β form is capable of more extensive hydrophobic interactions than the α-helical form. From the expected volume changes in the transition state (see above) it is possible to postulate a source for the compensation phenomena observed. Lumry and Rajender (1970) have proposed a mechanism for compensation behavior in terms of the two-state theory of water structure (Ives and Lemon, 1968) using the data of Walrafen (1968). The volume decrease of the polymer solutes on disruption of hydrophobic interactions and exposure of the hydrophobic side chains to water would be accommodated by expansion of the surrounding solvent, the expansion being mediated by the "hydrogen-bond making" process of the two-state theory of water. Walrafen's data (1968) give the values of ΔH, ΔS, and ΔV for "making one mole of hydrogen bonds in water" as −2.5 kcal, −8.5 eu, and 7 ml, respectively; thus $\Delta H/\Delta S = 300$°K, which is in good agreement with the value of T_c (302.5°K) found in this work. This is a viable interpretation of the source of compensation in the transitions observed herein and lends support to the mechanism proposed by Lumry and Rajender (1970).

On the basis of the experimental data and the above interpretations, it is possible to suggest a hypothesis for the transition and transition state. Undoubtedly the transition state will not involve hydrogen bonding and the positive enthalpy contribution to $\Delta H_{\alpha\rightarrow\beta}{}^{\pm}$ will add to the enthalpy of conformational unfolding. As the leucine content of the polymers increases the proportion of hydrophobic bonding increases in the helical form and thus will enhance the enthalpic stability of the helical form. However once the hydrogen-bonded backbone is broken or partially broken, the hydrophobic bonding is insufficient to maintain the helical form and the structure becomes more flexible and solvated. On the other hand, the β form which is capable of forming more and stronger hydrophobic interactions (Némethy and Scheraga, 1962a; Davidson and Fasman, 1967; Noguchi and Yang 1971) will contain a much larger enthalpic contribution from such interactions; these interactions are then sufficient to make the β form more stable than the helical form at elevated temperatures. The transition state would involve the helical form extended such that the hydrogen-bonded core would be solvated and the hydrophobic side-chain interaction would be separated sufficiently to satisfy the enthalpic and entropic contributions to the free energy of solvation.

The reverse transition from the β form → α helical form, at

277°K, is more difficult to obtain accurate kinetic data from, as the time scale required is much longer than for the $\alpha \rightarrow \beta$ transition. This frequently gives rise to the complication of aggregation and precipitation. An additional complication was the small buffering capacity of the polymer solution in 0.05 M KF. Even though the solutions were stored in a desiccator over sodium hydroxide flakes, the pH drifted down at a significant rate over the time scale of the experiments. It was necessary therefore to use 0.01 M boric acid as the solvent to increase the buffering capacity of the solution over the pH range of interest, which resulted in the pH drifting less than 0.02 pH unit during the experiments. The rate of the transconformation between the β and α forms, at 277°K, was found to be pH dependent. At the pH of the forward transition, pH 11.40, the rate for all the polymers was so slow that changes in the CD spectrum could not be detected after 24 hr. Therefore the chiroptical spectral changes with time as a function of pH were examined to determine the pH at which measureable rates could be obtained for all the polymers. The pH chosen was 10.6; at this pH, the helix is not completely uncharged; however, the CD spectra correspond to the completely helical forms. Therefore the thermodynamic parameters for the activation process in the $\beta \rightarrow \alpha$ transition contain contributions from charge interactions. But to a first approximation the effects can be assumed to be the same for all the polymers, as the degree of ionization, α, is similar at this pH for all three polymers (Snell and Fasman, 1972). These values are shown in Table IV.

Adequate data were obtained at pH 10.6, at 277°K, to give first-order rate constants, $k_{\beta \rightarrow \alpha}$, and thus values for $\Delta G_{\beta \rightarrow \alpha}^{\ddagger}$ listed in Table IV. The differences in $\Delta G_{\beta \rightarrow \alpha}^{\ddagger}$ between polymers is small and of doubtful significance but in the absence of data on $\Delta H_{\beta \rightarrow \alpha}^{\ddagger}$ and $\Delta S_{\beta \rightarrow \alpha}^{\ddagger}$ one cannot say that a compensation effect is observed for this transition. Evaluation of $\Delta G_{\alpha \rightarrow \beta}^{\ddagger}$ at 277°K allows computation of $\Delta G_{\beta \rightleftarrows \alpha}$ (277°K); it is clear that, at 277°K, as the leucine content of the polymers increases, the transition $\beta \rightarrow \alpha$ becomes less favorable. This is almost certainly due to the increased stability endowed on the β structure, relative to the α helix, by the increased proportion of strongly hydrophobic groups, *i.e.*, leucine. However the evaluation of $\Delta G_{\beta \rightleftarrows \alpha}$ (277°K) must be treated with reservation as the ionic strength of the aqueous solvent for the forward and reverse transitions is different; nevertheless, the trends can be expected to reflect the true situation. The value of $\Delta G_{\alpha \rightleftarrows \beta}$ (277°K) for poly(L-lysine) found herein (−90 cal mol residue^{-1}) is in fairly good agreement with the value of −62 cal mol^{-1} estimated at 298°K by Pederson *et al.* (1971) from the equilibrium free energy of the coil to β and coil to α transitions of poly(L-lysine) assuming the coil form as the transition site.

In conclusion, the intramolecular forces which govern the transitions observed in this work are hydrophobic interactions. These forces do not necessarily make a very large contribution to the observed thermodynamic parameters, but give a significantly negative contribution to otherwise positive quantities, and thus have a very noticeable and characteristic effect. The compensation phenomenon observed can be adequately explained in terms of volume changes associated with solvation of hydrophobic groups in the transition state. This work represents the first example of a comparative study of a series of polypeptides in the β conformation, thus allowing, through kinetic behavior, evaluation of the forces stabilizing such a structure and also a measure of the relative stability of these β conformations for this series in an aqueous environment. The relevance of this study to conformational changes in proteins is obvious and hopefully a better understanding of such protein transitions will be possible through this and further studies.

References

Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1972), *Methods Enzymol.* (in press).

Applequist, J. (1959), Ph.D. Thesis, Harvard University.

Applequist, J., and Doty, P. (1958), Abstracts, 133rd National Meeting of the American Chemical Society, San Francisco, Calif., April, p 32a.

Applequist, J., and Doty, P. (1962), *in* Polyamino Acids, Polypeptides and Proteins, Stahmann, M., Ed., Madison, Wis., University of Wisconsin Press, p 161.

Astbury, W. T., Dickinson, S., and Bailey, K. (1935), *Biochem. J. 29*, 2351.

Blake, C. C. F., Koenig, D. F., Maia, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1965), *Nature (London) 206*, 757.

Blout, E. R., and Shechter, E. (1963), *Biopolymers 1*, 565.

Bradbury, E. M., Brown, L., Downie, A. R., Elliot, A., Fraser, R. D. B., Hanby, W. E., and McDonald, T. R. R. (1960), *J. Mol. Biol. 2*, 276.

Brandts, J. F. (1969), *in* Biological Macromolecules, Vol. 2, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 213.

Conio, G., Patrone, E., and Salaris, F. (1971), *Macromolecules 4*, 283.

Davidson, B., and Fasman, G. D. (1967), *Biochemistry 6*, 1616.

Davidson, B., Tooney, N., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 156.

Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968), *Nature (London) 218*, 929.

Fasman, G. D. (1967), *in* Biological Macromolecules, Vol. I, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 499.

Fasman, G. D., Idelson, M., and Blout, E. R. (1961), *J. Amer. Chem. Soc. 83*, 709.

Fasman, G. D., Lindblow, C., and Bodenheimer, E. (1964), *Biochemistry 3*, 155.

Fasman, G. D., and Potter, J. (1967), *Biochem. Biophys. Res. Commun. 27*, 209.

Fieser, L. F. (1941), Experiments in Organic Chemistry, 2nd ed, Boston, Mass., D. C. Heath and Co., p 361.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley.

Glasstone, S., Laidler, K. J., and Eyring, H. (1941), The Theory of Rate Processes, 1st ed, New York, N. Y., McGraw-Hill.

Goodman, M., Naider, F., and Toniolo, C. (1971), *Biopolymers 10*, 1719.

Ikeda, S., and Fasman, G. D. (1967), *J. Mol. Biol. 30*, 491.

Ikeda, S., and Imae, T. (1972), *Biopolymers 11*, 493.

Imae, T., and Ikeda, S. (1972), *Biopolymers 11*, 509.

Ives, D., and Lemon, T. (1968), *Roy. Inst. Chem. Rev. 1* 62.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Leffler, J., and Grunwald, E. (1963), Rates and Equilibria of Organic Reactions, New York, N. Y., Wiley.

Li, L. K., and Spector, A. (1969), *J. Amer. Chem. Soc. 91*, 220.

Lumry, R., and Eyring, H. (1954), *J. Phys. Chem. 58*, 110.

Lumry, R., and Rajender, S. (1970), *Biopolymers 9*, 1125.

Maeda, H., and Ikeda, S. (1971a), *Biopolymers 10*, 1635.

Maeda, H., and Ikeda, S. (1971b), *Biopolymers 10*, 2525.

Makino, S., and Noguchi, H. (1971), *Biopolymers 10*, 1253.

Makino, S., and Sugai, S. (1970), *Biopolymers 9*, 1049.

Myer, F., and Barnard, D. (1971), *Arch. Biochem. Biophys. 143*, 116.
Némethy, G., and Scheraga, H. A. (1962a), *J. Phys. Chem. 66*, 1773.
Némethy, G., and Scheraga, H. A. (1962b), *J. Chem. Phys. 36*, 3401.
Noguchi, H., and Yang, J. T. (1971), *Biopolymers 10*, 2569.
Parker, K. D., and Rudall, K. M. (1957), *Nature (London) 179*, 905.
Pauling, L., and Corey, R. B. (1951), *Proc. Nat. Acad. Sci. U. S. 37*, 729.
Pederson, D., Gabriel, D., and Hermans, J., Jr. (1971), *Biopolymers 10*, 2133.
Quadrifoglio, F., and Urry, D. W. (1968), *J. Amer. Chem. Soc. 90*, 2760.
Rosenheck, K., and Doty, P. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1775.
Rossman, M. G., Adams, M. J., Buehner, M., Ford, G. C., Hackert, M. L., Lantz, P. J., Jr., McPherson, A., Jr., Schevitz, R. W., and Smiley, I. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 179.
Sarkar, P. K., and Doty, P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 981.
Scheraga, H. A., Némethy, G., and Steinberg, I. Z. (1962), *J. Biol. Chem. 237*, 2506.
Senti, F. R., Eddy, C. R., and Nutting, G. C. (1943), *J. Amer. Chem. Soc. 65*, 2473.
Snell, C. R., and Fasman, G. D. (1972), *Biopolymers 11*, 1723.
Spackman, D. H., Stein, H. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Tooney, N., and Fasman, G. D. (1968), *J. Mol. Biol. 36*, 355.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., and Davidson, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 163.
Walrafen, G. (1968), *in* Equilibria and Reaction in Hydrogen Bonded Solvent Systems, Covington, A., and Jones, P., Ed., London, Taylor and Frances, p 9.
Widmer, U., and Lorenzi, G. P. (1971), *Chimia 25*, 236.
Wooley, S.-Y. C., and Holzwarth, G. (1970), *Biochemistry 9*, 3604.

Bovine β-Lactoglobulins in Urea Solution. Denaturation at pH 5.2 and 3.5†

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ABSTRACT: The denaturation reactions of bovine β-lactoglobulins A, B, and C in urea solution at pH 5.2 and 3.5 are compared. The optical rotation and viscosity, and their rate of change with time, are strongly dependent on urea concentration. In 7 M urea at pH 5.2 the optical rotation change may be broken down by kinetic analysis into a primary and a secondary stage. The primary change does not follow simple first-order kinetics, but may be decribed by the sum of two exponential terms. Its half-time decreases slightly with increasing protein concentration. The primary denaturation is not a two-state process and involves sequential and/or parallel reactions. In this stage the rotation change is largely reversible but on prolonged reaction the extent of reversibility decreases with increasing reaction time, the kinetics of renaturation always being complex. Each variant is more readily unfolded by urea at pH 3.5 than at pH 5.2. The change in optical rotation with time, at pH 3.5 and urea concentrations where the reaction is sufficiently slow for kinetic measurements to be made, consists of a rapid primary and a much slower secondary stage. The primary change at 578 nm is sometimes apparent first order, but that at 260–300 nm is not first order. The presence of stable intermediates at the end of the primary stage is demonstrated by analysis of ORD curves. On prolonged reaction irreversible products, due to -SH/-SS- interchange, are produced at pH 3.5, but to less extent than at pH 5.2. The order of kinetic and thermodynamic stability of the variants is determined, and a general mechanism for their behavior in urea is proposed.

The work described here arose out of two series of investigations in this laboratory. One series is concerned with the chemical evolution of individual milk and blood proteins (McKenzie, 1967, 1971). The other is concerned with comparative studies of the denaturation and aggregation of proteins under a variety of conditions with the aim of gaining a better understanding of the mechanism of these processes and hence of some aspects of protein structure (McKenzie and Ralston, 1971). The rationale of the investigations is that the change with time of several experimental parameters, sensitive to different properties of the protein molecule, is studied under varying conditions. The effects of denaturant and its concentration, pH, temperature, and group specific reagents, and the reversibility of the reactions are determined. If any theory of protein denaturation ("unfolding") is generally valid it should be applicable to proteins containing cysteine and cystine residues as well as to those not containing them. Thus both groups of proteins are being investigated in this laboratory. The unfolding of the former group is

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