course of the experiment. Olsen (1976) has, in fact, shown that two forms (not R and T) are produced on addition of IHP to methemoglobin and that they are not rapidly interconvertible.

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Sedimentation Studies of the Reversible Dimer–Tetramer Transition Kinetics of Concanavalin A†

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ABSTRACT: The self-association of concanavalin A in solution at pH 7 was analyzed at different temperatures (4, 15, 23, 31 °C) using the analytical band-sedimentation technique linked with a computer simulation method. In the concentration range investigated, which is close to that used in studying the agglutinating and binding properties of concanavalin A, the polymerization of this lectin depends on temperature; up to 23 °C, concanavalin A is essentially subject to a reversible dimer–tetramer transition; above this temperature, the specific dimer–dimer interaction is disturbed by isodesmic associations, which are negligible at lower temperatures. The kinetic parameters of the dimer–tetramer transition were determined at 4, 15, and 23 °C: for 4 °C $\leq t \leq$ 23 °C the equilibrium constant K varied from 1.9×10^4 M^{-1} to 6.9×10^5 M^{-1}, and the relaxation time values were very large varying from 6500 to 440 s which indicated that important structural changes were taking place. The experimental results were used to calculate values for the following thermodynamic constants of the associative reaction: ΔG_0, ΔH_0, ΔS_0, and $\Delta G_{\pm}^{\ddagger}$, $\Delta H_{\pm}^{\ddagger}$, $\Delta S_{\pm}^{\ddagger}$. This reaction is essentially characterized by large and positive values of ΔH_0 (25–39 kcal) and ΔS_0 (118–160 cal/deg). These findings led us to conclude that the two dimers associate through a large hydrophobic binding region. This conclusion is supported by previous crystallographic results (Reeke, G. N., Jr., Becker, J. W., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1525, 1547) and allows us to propose a model for this interaction process.

The primary sequence and the three-dimensional structure of concanavalin A have been well established (Wang et al., 1975; Cunningham et al., 1975; Becker et al., 1975; Reeke et al., 1975). Moreover, the quaternary structure of concanavalin A in solution is pH and temperature dependent: at physiological pH (pH 7.2) and 0 °C the protein is a dimer, but at 37 °C it is a tetramer. At pH 5.6, in the same temperature range, it is always a dimer (McKenzie et al., 1972; Huet et al., 1974; Huet, 1975). Nevertheless, the thermodynamic and kinetic characteristics of the transition between these two forms of the molecule and between them and possibly more polymerized states have not been studied. Previous analytical centrifugation studies on the variation of the concanavalin A sedimentation

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coefficient with temperature and pH suggested that there is a reversible dimer–tetramer equilibrium (Huet et al., 1974; Huet, 1975).

In the present work, using the same experimental conditions of pH and concentration as those used in cellular studies, we analyzed the polymerization of concanavalin A and determined its kinetic and thermodynamic parameters, using the analytical band-sedimentation technique and a computer simulation method developed in our laboratory (Claverie et al., 1975; Cohen and Claverie, 1975; Claverie, 1976).

Experimental Procedures

Chemicals. Concanavalin A from Jack bean meal (Con A),[1] three time crystallized and lyophilized, was purchased from Miles Yeda (Israel). Solutions of Con A were prepared in 0.01 M phosphate buffer at pH 7.2 containing 0.14 M NaCl and 2.6×10^{-3} M KCl (phosphate buffered saline). The final concentration of the protein solutions was estimated by spectrophotometry at 280 nm, on the basis of $A_{1cm}^{1\%} = 12.4$ absorbancy units (Kalb and Lustig, 1968). All the buffered solutions used in analyses were prepared with Merck chemical products.

Analytical Ultracentrifugation: Band-Sedimentation Method. Ultracentrifugation analyses were performed in a Beckman Spinco Model E ultracentrifuge equipped with a standard monochromator, a RTIC temperature control unit, and a special split-beam scanner system connected by interface to a Honeywell H.516 computer, the MaD system (Cohen et al., 1976). With this system, which involves an absorption optic device, we are able to measure optical densities lower than 0.05 absorbancy unit with a precision better than 10^{-3} absorbancy units. We could therefore use the band-sedimentation method with initial concentrations down to 0.2 mg/mL. Compared with the boundary-sedimentation method, this technique is more sensitive to molecular polydispersity and is, thus, more suitable for studying the equilibrium between dimeric and tetrameric forms.

The experiments were performed at a wavelength of 280 nm and a speed of 52 000 rev/min. Double-sector 12-mm charcoal filled epon band-forming centerpieces were used; the two sectors were filled with 0.35 mL of a 0.01 M phosphate buffer at pH 7.2, to which 0.5 M NaCl and 2.6×10^{-3} M KCl had been added. Ten microliters of a Con A solution was layered over this buffered solution in one sector of the centrifugation cell and 10 μL of phosphate-buffered saline in the other sector. After stabilization of two Con A solutions (0.5 and 2 mg/mL) at one of the four experimental temperatures (4, 15, 23, 31 °C), we centrifuged them at the same temperature in the same rotor. Some 30 min after the beginning of the centrifugation, the dilution factor of the protein band was about 5, and during the remaining duration of the centrifugation it was about 2; thus the effective concentration range studied during our experiments was 0.05–0.4 mg/mL. We verified, with a cary 15 spectrophotometer that the UV spectrum of the Con A molecule was not modified by increasing the temperature from 4 °C up to 31 °C. The sedimentation coefficients were obtained from plots of log r vs. time (r = radial abscissas of the mass-center of the protein band) (Fujita, 1975).

Method of Kinetic Analysis. The kinetic studies were performed with the simulation method previously described (Claverie, 1976). According to this method, we analyzed the Con A equilibrium by comparing our band-sedimentation results with predictions of a reversible dimer–tetramer (D–T) transition model according to the reaction $2D\ (k^+) \rightleftarrows (k^-)\ T$; the kinetic equations of this transition are:

$$\frac{d[D]}{dt} = 2(k^-[T] - k^+[D]^2)$$

$$\frac{d[T]}{dt} = -\frac{1}{2}\frac{d[D]}{dt}$$

where [D] and [T] are the molar concentrations of the dimeric and the tetrameric forms; k^+ and k^- are the kinetic constants of the chemical reaction. To simulate the sedimentation of such a transition we must resolve the following Lamm equations system:

$$\frac{\partial c_i}{\partial t} + \frac{1}{r}\frac{\partial}{\partial r}\left(s_i\omega^2 r^2 c_i - rD_i\frac{\partial c_i}{\partial r}\right) = f_i$$

with the usual boundary conditions:

$$s_i\omega^2 r_m c_i - D_i\left[\frac{\partial c_i}{\partial r}\right]_{r_m} = 0$$

$$s_i\omega^2 r_b c_i - D_i\left[\frac{\partial c_i}{\partial r}\right]_{r_b} = 0$$

where $i = 1$ or 2, corresponds respectively to the dimeric and the tetrameric forms of Con A. s_i and D_i are respectively the sedimentation and the diffusion coefficients, c_i is the concentration of the ith species, ω is the centrifugation angular velocity, r_m and r_b are the meniscus and the bottom radial abscissas; the coupling terms f_i are:

$$f_1 = 2[-k^+(c_1)^2 + k^-c_2]$$

$$f_2 = -\frac{1}{2}f_1$$

Finally, the initial conditions corresponding to the band sedimentation must be added to the preceding equations; they are:

$$c_i(0,r) = c_i(0);\ r_m \leqslant r \leqslant r_m + \delta$$

$$c_i(0,r) = 0;\ r_m + \delta < r \leqslant r_b$$

where δ is the initial thickness of the protein layer. The numerical solution of such a problem has been described elsewhere (Cohen and Claverie, 1975). To define the molecular transition of Con A, we evaluated the kinetic constants k^+ and k^- by the intermediate of the usual equilibrium constant $K = k^+/k^-$ and the relaxation time $\tau = (4k^+[D]_e + k^-)^{-1}$, where $[D]_e$ is the equilibrium concentration of the dimer. For the present purpose, these two independent parameters, K and τ, are more descriptive of the equilibrium than are the k^+ and k^- constants. Values of K and τ appropriate to the experimental results were obtained by a trial and error process using a computer. Trial values of K and τ, at given temperature and initial concentration, were used to perform simulations from which we calculated values of the weight-average sedimentation coefficient $\bar{s}_w$ and the second moment of the protein band, σ^2, at a given time. These $\bar{s}_w$ and σ^2 values were then compared with the experimental results, and the estimates for K and τ altered to improve the estimate until the best agreement was obtained between the calculated and experimental values of $\bar{s}_w$ (within 3%) and σ (within 8%). The $\bar{s}_w$ value gives an indication of the position, and the σ^2 value of the shape of the band. Using this method we determined the K and τ values and hence the corresponding k^+ and k^- constants for the lower of the two initial Con A concentrations (0.5 mg/mL); these values of the kinetic constants k^+ and k^- were then used for the simulation analysis of the results obtained with the higher Con A con-

[1] Abbreviation used: Con A, concanavalin A.

TABLE I: Sedimentation Coefficient Values of the Dimeric and Tetrameric Forms of Con A Molecule at the Experimental Temperatures Studied.[a]

t (°C)	s_t (S)	
	Dimer	Tetramer
4	2.20	3.61
15	2.98	5.02
23	3.60	6.02
31	4.27	7.08

[a] These s_t values were estimated from present and previous experimental results (see text) and are used as input data for the simulation analyses.

centration (2 mg/mL), and, in this way, the transition model was checked. For the simulation analysis, rather than taking values from the literature, we used values of sedimentation coefficients (s_t) of the dimer and the tetramer obtained from present band-sedimentation experiments at 4 °C (when only dimer is present) and from previous boundary-sedimentation experiments at 37 °C (where the sedimentation coefficient measured is that of the tetramer) (Huet, 1975); the values thus obtained are listed in Table I. From these values we also recalculated the corresponding diffusion coefficients, D_t, taking a molar mass of 51 150 daltons for the dimeric form (Reeke et al., 1975) and a partial specific volume constant equal to 0.73 mL/g (Agrawal and Golstein, 1968). The sedimentation and diffusion coefficients of the two species, corrected to standard conditions (20 °C and water as solvent) are $s_{20,w} = 3.50$ S, $D_{20,w} = 6.20 \times 10^{-7}$ cm^2/s for the dimer and $s_{20,w} = 5.85$ S, $D_{20,w} = 5.20 \times 10^{-7}$ cm^2/s for the tetramer; these values are in good agreement with those obtained by other workers (McCubbin and Kay, 1971; McKenzie et al., 1972).

Results

Ultracentrifugation Analyses. The weight-average sedimentation coefficient values obtained with 0.5 and 2 mg/mL initial protein concentrations, at 4 °C, 15 °C, 23 °C, and 31 °C, are listed in Table II (the values are not corrected to standard conditions). At 4 °C, the $\bar{s}_w$ value increases with the total concentration of Con A; at 0.5 mg/mL the molecule is a dimer ($\bar{s}_w = 2.21$ S) whereas, at 2 mg/mL, the value obtained ($\bar{s}_w = 2.53$ S) reveals the presence of a tetrameric form in the Con A solution; the same phenomenon is observed at all the temperatures studied; in addition it should be noted that the sedimentation profiles are never bimodal (Figures 1–2). These results confirm the hypothesis that there is an equilibrium between the dimeric and the tetrameric forms of the Con A molecule which depends on temperature; furthermore, the results suggest that the transition between the two forms takes place within a shorter time scale than the duration of the centrifugation.

Simulation Analyses: The Kinetic Parameters of the Con A Transition (Table III). Using the procedure described in Experimental Procedures, we tested the validity of the transition model adopted (dimer ⇄ tetramer) for the temperatures and the concentrations studied, and determined the corresponding equilibrium constants and relaxation times. At 4 °C, the correspondence between the experimental (exp) and simulated (sim) profiles is as good for 2 mg/mL as it is for 0.5 mg/mL (the data obtained are comparable to those shown in Figures 1A and 1B); the experimental and simulated values of the second moment of the band (σ^2_{exp} and σ^2_{sim}) are very close [$(\sigma_{exp} - \sigma_{sim})/\sigma = \Delta\sigma/\sigma = 8\%$ at 0.5 mg/mL, and 4% at 2 mg/mL] as are those of the sedimentation coefficient

TABLE II: Experimental (exp) and Simulated (sim) Weight-Average Sedimentation Coefficients of Con A in Solution for the Best Fit at Various Temperatures and Concentrations.

t (°C)	c_0 (mg/mL)[a]	$\bar{s}_w$(exp) (S)	$\bar{s}_w$(sim) (S)	$\Delta s/s$ (%)
4	0.5	2.21	2.38	7.5
	2.0	2.53	2.58	2.0
15	0.5	3.70	3.65	1.4
	2.0	4.05	3.93	3.0
23	0.5	4.85	4.84	0.2
	2.0	5.21	5.20	0.2
31	0.5	6.17	nd[b]	
	2.0	6.73	nd	

[a] c_0 is the initial concentration of Con A layered over the buffer solution at the beginning of the band sedimentation. The effective concentration will be between $c_0/5$ and $c_0/10$. [b] nd, not determinable.

TABLE III: Values of the Kinetic Parameters of the Reversible Dimer–Tetramer Transition of the Con A Molecule at Various Temperatures[a] and Concentrations.[b]

t (°C)	c_0 (mg/mL)	k^+ (M^{-1} s^{-1})	k^- (s^{-1})	K (M^{-1})	τ (s)[c]
4	0.5	2	1.1×10^{-4}	1.9×10^4	6500
	2.0				3700
15	0.5	26	2.4×10^{-4}	1.1×10^5	1400
	2.0				750
23	0.5	215	3.1×10^{-4}	6.9×10^5	440
	2.0				nd

[a] In this table as in Tables IV and V, we have not considered t = 31 °C since the dimer ⇄ tetramer equilibrium model is no longer applicable to the experimental results and the kinetic parameters are no longer determinable (nd). [b] c_0 is the initial protein concentration. [c] k^+, k^-, and K are not dependent on concentration but τ is.

($\Delta s/s = 7.5\%$ at 0.5 mg/mL and 2% at 2 mg/mL) (Table II). At this temperature the Con A molecule is essentially in the dimeric form and our fitting method is not very precise; thus the precision for the equilibrium constant and the relaxation time values were both within ±15%. At 15 as at 4 °C, with 0.5 and 2 mg/mL, we obtained a good agreement between predictions of the model and the experimental results ($\Delta\sigma/\sigma = 8\%$ and $\Delta s/s = 2\%$) (Figures 1A and 1B) (Table II); the dimer and the tetramer concentrations are similar at this temperature and we can obtain a better evaluation of the equilibrium parameters (±10%). At both temperatures, the systematic deviations of the simulated profiles from the experimental one can be explained by a necessarily imperfect evaluation of the initial conditions in band sedimentation (i.e., the exact thickness of the protein layer, the vibrations and mini-convections occurring at the beginning of a centrifugation); the introduction of either higher polymers or incompetent dimeric species (McKenzie and Sawyer, 1973) always gave a worthy fit between experimental and simulated results (data not shown). At 23 °C, for the lower concentration (0.5 mg/mL), there was still a good agreement between the experimental results and predictions of the model ($\Delta\sigma/\sigma = 1.6\%$ and $\Delta s/s = 0.2\%$), but the kinetic constants k^+ and k^- obtained did not fit the results obtained at the higher concentration (2 mg/mL): the experimental broadening of the protein band was larger than the

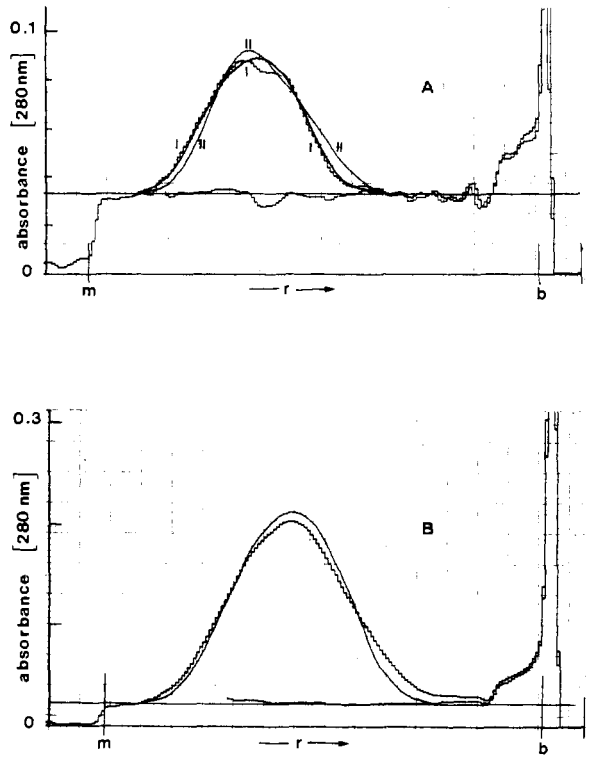

FIGURE 1: Comparison of experimental and simulated sedimentation profiles of a Con A solution at 15 °C for the best fit. The two-step curves are respectively the experimental profile of the protein band as obtained with the MaD system and the experimental base-line obtained when the protein band was still near the meniscus. The continuous curve (I) is the experimental profile after subtraction of the base-line; the continuous curve (II) is the simulated profile. (A) C_0 = 0.5 mg/mL, t = 5220 s; (B) C_0 = 2 mg/mL, t = 4970 s; (where C_0 is the initial Con A concentration layered onto the buffered solution at the beginning of the centrifugation; t is the centrifugation time at which the experimental profile was obtained).

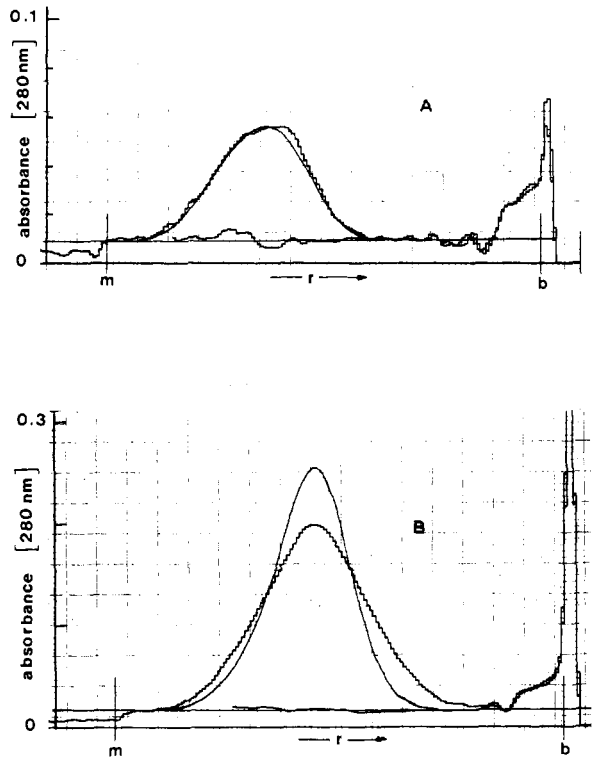

FIGURE 2: Comparison of experimental and simulated sedimentation profiles of a Con A solution at 23 °C for the best fit. (A) C_0 = 0.5 mg/mL, t = 4110 s; (B) C_0 = 2 mg/mL, t = 4410 s;

simulated one ($\Delta\sigma/\sigma$ = 32%) though the corresponding $\bar{s}_w$ values remained identical ($\Delta s/s$ = 0.2%) (Figures 2A-2B) (Table II). The deviation from predictions of the model could be explained by the presence of a small amount of higher molecular weight species (cf. Discussion). Finally, at 31 °C, it was impossible, at both concentrations, to obtain K and τ values giving a good fit between our experimental profiles and those obtained from the equilibrium model chosen. At this temperature, it appears that the considerable broadening of the protein band cannot be explained by the sedimentation of the tetramer only, which should be the predominant species according to our model.

Simulation Analyses: The Thermodynamic Parameters of the Con A Transition. For a better understanding of this transition we determined the standard thermodynamic parameters ΔG_0, ΔH_0, ΔS_0, and the activation parameters $\Delta G_\pm^\ddagger$, $\Delta H_\pm^\ddagger$, and $\Delta S_\pm^\ddagger$ for the association (+) and the dissociation (−) reactions; their values are listed in Tables IV and V. These calculations were made from the equilibrium constant K and the constants k^+ and k^- obtained above, according to the following usual formula (Mahler and Cordes, 1966):

$$K = \exp(-\Delta G_0/RT)$$

$$k = \frac{k_B T}{\hbar} \exp(-\Delta G^\ddagger/RT)$$

where k is k^+ or k^-, k_B is the Boltzmann constant, $\hbar$ the Planck constant, and T the absolute temperature (K). In all the calculations K, k^+, and k^- are expressed with respect to molar fractions rather than molarity.

Discussion

At neutral pH, under experimental conditions commonly used to study the agglutinating and binding properties of Con A, we have verified that the molecular transition of this lectin is essentially between the dimer and the tetramer up to 23 °C. Above this temperature, the experimental data no longer fit the patterns predicted by the dimer-tetramer equilibrium model. Nor do simulations attempted with dimer ⇄ tetramer ⇄ hexamer or dimer ⇄ tetramer ⇄ octamer transitions correlate any better at 31 °C with the experimental findings. Furthermore, we did not observe any time dependence of the sedimentation coefficient of Con A and no variation of its value could be detected when various amounts of solvent were layered over a Con A solution (0.2 mg/mL) in a synthetic boundary cell (Harrington and Kegeles, 1973). As we established by simulation assays (data not shown) that a significant pressure effect on the band-sedimentation patterns should be detected, in our experimental conditions, by synthetic boundary cell sedimentation experiments, we, thus, conclude that there

TABLE IV: Effect of Temperature on the Standard Thermodynamic Parameters of the Dimer ⇄ Tetramer Equilibrium of Con A in Solution.

t (°C)	ΔG_0 (kcal)[a]	ΔH_0 (kcal)[b]	ΔS_0 (cal/deg)
4	−7.6		
		25	118
15	−8.9		
		39	160
23	−10.0		

[a] ΔG_0 is measured at a given temperature; it is obtained with a precision of ±1-2%. [b] ΔH_0 and ΔS_0 are estimated between two temperatures; they are obtained with a precision of ±15% for the interval (4-15 °C) and ±10% for the interval (15-23 °C).

TABLE V: Effect of Temperature on the Activation Parameters for the D → T Reaction (+) and the T → D Reaction (−).[a]

t (°C)	$\Delta G_+^{\ddagger}$ (kcal)	$\Delta G_-^{\ddagger}$ (kcal)	$\Delta H_+^{\ddagger}$ (kcal)	$\Delta H_-^{\ddagger}$ (kcal)	$\Delta S_+^{\ddagger}$ (cal/deg)	$\Delta S_-^{\ddagger}$ (cal/deg)
4	13.6	21.0				
			36	10.0	82	−37
15	12.6	21.6				
			44	4.5	100	−57
23	11.8	22.0				

[a] $\Delta G_{\pm}^{\ddagger}$ values are estimated at a given temperature with a precision of ±1-2%; $\Delta H_{\pm}^{\ddagger}$ and $\Delta S_{\pm}^{\ddagger}$ values are estimated between two temperatures with a precision of ±15% for the interval (4-15 °C) and ±10% for the interval (15-23 °C).

is no significant and detectable effect due to pressure which influenced the interpretation of our results. Therefore, the experimental patterns observed at 31 °C could be explained by isodesmic associations and the production of aggregates. Such aggregate formation has been previously observed at neutral pH, low ionic strength (<0.1), and high Con A concentrations (>5 mg/mL) (Agrawal and Golstein, 1968; McKenzie et al., 1972). Moreover, the crystallographic results which show that the tetrameric form of the Con A molecule is a closed symmetrical structure excluding further specific associations is in agreement with the existence of such isodesmic associations (Hardman et al., 1971; Edelman et al., 1972; Reeke et al., 1975). The present results show that there is a temperature and concentration range in which the dimer-tetramer transition model holds true (i.e., the proportion of higher polymerized forms is negligible). They also show that, beyond this range, the extent of aggregation becomes large and the dimer-tetramer equilibrium model is no longer sufficient to describe the experimental results.

Where the experimental and simulated patterns are consistent with the dimer ⇄ tetramer model, we were able to estimate the equilibrium parameters with a precision between 10% and 15% depending on the temperature, i.e., on the relative proportions of the dimeric and tetrameric forms. We obtained very large values of relaxation time; such high values have been already reported in the case of other molecular systems (Hofer and Krystek, 1975; Ip et al., 1976); they signify important structural changes which we have tried to analyze by determining the thermodynamic parameters of the associative reaction. The self-association of the Con A molecule is an entropy-driven one ($T\Delta S_0 > \Delta H_0 > 0$) typical for interactions of hydrophobic nature (Scheraga, 1963). This is in agreement with the crystallographic studies of Reeke et al. (1975) which indicate that there is a large pleated sheet zone involving salt links, hydrogen bonds and also, most importantly, numerous hydrophobic bonds. These data and the experimentally determined activation parameters lead us to propose that Con A tetramer formation could be mainly controlled by the following interaction process. In the free dimer, the large β structure (which is the hydrophobic binding region between two dimers) is found entirely on the molecular surface; it is completely exposed to solvent and thus covered with an organized layer of water molecules exchanging numerous hydrogen bonds. When the collision between two free dimers occurs, the two corresponding layers of water molecules are partly disordered allowing the formation of an "activated complex" in which the surface of the hydrophobic region is partially exposed due to displacement of water molecules. This exposure of both association surfaces should be accompanied by the fusion of parts of the two structured water layers. In the temperature range 4-15 °C, for example, this could require the 36 kcal of activation enthalpy ($\Delta H_+^{\ddagger}$), measured experimentally, in order to break the hydrogen bonds. At the same time, these changes could involve an increase of entropy ($\Delta S_+^{\ddagger}$) by the 82 cal/deg, found in these experiments. Destruction of the water layers allows formation of the tetramer. During this final step, hydrogen bonds as well as salt links between the lateral chains of the two dimers could be exchanged, thus explaining the observed negative increase of enthalpy ($-\Delta H_-^{\ddagger}$) by −10 kcal and of entropy ($-\Delta S_-^{\ddagger}$) by an additional 37 cal/deg. This simple model for Con A tetramer formation is consistent not only with the crystallographic results mentioned above, but also with the hydrophobic nature of the interactions of this lectin with other proteins in affinity chromatography (Davey et al., 1976).

In conclusion, our results demonstrate clearly that the polymerization of the Con A molecule depends on temperature. In our experimental conditions which are precisely those used in cell surface studies of Con A as structural probe, this lectin exists simultaneously in the dimeric and tetrameric forms, an observation which must always be considered in the interpretation of experiments on the binding of Con A to cell surfaces.

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Thermodynamics of the Denaturation of Pepsinogen by Urea†

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ABSTRACT: The denaturation of swine pepsinogen has been studied as a function of urea concentration, pH, and temperature. The unfolding of the protein by urea has been found to be fully reversible under different conditions of pH, temperature, and denaturant concentration. Kinetic experiments have shown that the transition shows two-state behavior at 25 °C in the pH range 6-8 covered in this study. Analysis of the equilibrium data obtained at 25 °C according to Tanford (Tanford, C. (1970), *Adv. Protein Chem. 24,* 1) and Pace (Pace, N. C. (1975), *Crit. Rev. Biochem. 3,* 1) leads to the conclusion that the free energy of stabilization of native pepsinogen, relative to the denatured state, under physiological conditions, is only 6-12 kcal mol^{-1}. The temperature dependence of the equilibrium constant for the unfolding of pepsinogen by urea in the range 20-50 °C at pH 8.0 can be described by assigning the following values of thermodynamic parameters for the denaturation at 25 °C: ΔH = 31.5 kcal mol^{-1}; ΔS = 105 cal deg^{-1} mol^{-1}; and ΔC_p = 5215 cal deg^{-1} mol^{-1}.

We have been interested for some time in interactions in the swine pepsinogen molecule and the bearing of these on its mechanism of activation to pepsin (McPhie, 1975). Perlmann (1963) showed that high concentrations of urea transformed the zymogen into a form which could not be activated at low pH and that removal of urea restored the potential activity to 90% of the native value. Edelhoch and co-workers (1965) studied the kinetics of this transition and also showed its product to be an open, expanded form of the molecule (Frattali et al., 1965). More recently, Lapanje (1969) interpreted his measurements of the intrinsic viscosity of pepsinogen solutions in 8 M urea to indicate that the protein exists as a cross-linked random coil under these conditions. We report here a more thorough investigation of the thermodynamics of the unfolding of pepsinogen by urea, using well known methods (Tanford, 1968, 1970). We are interested in the structural determinants which give the zymogen greatly enhanced stability at neutral pH, as compared with pepsin.

Experimental Procedure

Pepsinogen (lots PG34D903 and PG36S810) obtained from Worthington Biochemical Corp. and UltraPure samples of urea purchased from Schwarz/Mann were used without further purification. Other chemicals were analytical grade.

Absorption spectra were measured in a Cary 15 spectrophotometer using tandem thermostated cells. Protein solutions for the equilibrium and kinetic studies of the denaturation of pepsinogen were prepared as follows. For denaturation experiments, known amounts of the stock protein, buffer, and urea solutions at the same pH were mixed in a 5-mL volumetric flask and incubated for a period that was found to be sufficient by kinetic experiments for the completion of the reaction. A similar procedure was employed in preparing the protein solutions for renaturation experiments with the only exception that pepsinogen was first denatured in concentrated urea solution and then diluted with buffer. Sodium phosphate buffer of ionic strength 0.15 M was used throughout this study unless stated otherwise. Pepsinogen concentration was determined using a value of 5.1×10^4 for the molar extinction of the native protein at 278 nm. Proteolytic activity was estimated with a 25 mg/mL solution of hemoglobin in 0.1 M hydrochloric acid, as described previously (McPhie, 1975).

The kinetics of urea denaturation at 25 °C were studied at pH values 6, 7, and 8 and at different urea concentrations. For denaturation experiments, a known volume of urea solution in phosphate buffer was taken in a cell kept in the spectrophotometer. To this, 100 to 200 μL of a concentrated solution of pepsinogen was added with the help of an "adder-mixer." The rate of denaturation was determined by following the change in absorbance at 292 nm immediately after mixing the two solutions in the cell. The rate of renaturation was measured similarly except that a concentrated solution of pepsinogen in urea of desired concentration was added to a known volume of buffer. Thus it was possible to record the progress of the denaturation and renaturation reactions from 3 s after mixing. The first-order rate constants were computed from standard

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