Thermodynamics of Concanavalin A Dimer–Tetramer Self-Association: Sedimentation Equilibrium Studies†

Donald F. Senear‡ and David C. Teller*

ABSTRACT: The effects of temperature and pH on the reversible dimer–tetramer association of concanavalin A were studied by the high-speed sedimentation equilibrium technique. Both commercial and highly purified preparations of concanavalin A were used. Equilibrium constants were analyzed as a Wyman linked function by using truncated van't Hoff temperature dependence. In the concentration range 0.1–3.0 mg/mL, from 5 to 35 °C and between pH 5.5 and pH 7.5 at 0.5 M ionic strength, only dimer and tetramer species were present in both preparations. For purified concanavalin A, association constants ranged from 1.5×10^3 to 8.0×10^7 M^{-1}. Constants for our commercial preparation were ~10-fold lower due to the decreased competency of some subunits to self-associate. From the fit of the Wyman model to the experimental data, $\Delta G°$, $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ were calculated for the association and association-linked ionization reactions. From the values of the ionization thermodynamic parameters, the association is governed by the ionization of a histidine side chain on each subunit, either histidine-51 or histidine-121. The association is characterized by large entropy (66.3 cal·mol^{-1}·deg^{-1} at 25 °C) and heat capacity (−821 cal·mol^{-1}·deg^{-1}) changes in accordance with the large hydrophobic association surface observed in crystallographic studies [Reeke, G. N., Jr., Becker, J. W., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1525–1547]. In addition, there is a large enthalpy change (10.4 kcal·mol^{-1} at 25 °C). We propose a model for the interaction based on a more detailed thermodynamic description than was obtained in an earlier, incomplete study [Huet, M., & Clavarie, J. M. (1978) *Biochemistry 17*, 236–241].

Interest in the unusual biological properties of the jack bean lectin concanavalin A (Con A)[1] has made it the subject of intense biochemical study. The primary and three-dimensional structures are known (Cunningham et al., 1975; Becker et al., 1975; Hardman & Ainsworth, 1972), and the interactions between the subunits that form the crystalline quaternary structure have been well described (Reeke et al., 1975). Although it crystallizes as a tetramer of identical 25 500 molecular weight protomers, some of which are fragmented (Kalb & Lustig, 1968; Wang et al., 1971), the solution quaternary structure is pH and temperature dependent. Con A has been reported to be a dimer at pH 5.5 and a tetramer at pH >7 (McKenzie et al., 1972; Huet, 1975). An analysis of this self-polymerization has shown that a dimer–tetramer equilibrium exists (Huet & Clavarie, 1978).

This equilibrium is of interest because some biological properties of Con A have been shown to depend on the valency of Con A for saccharide ligands [e.g., Gunther et al. (1973) and Yasaka & Kambara (1979)]. It is also of interest because it provides a model for the energetics involved in maintaining the folded structures of globular proteins. Chothia (Chothia & Janin, 1976) has shown that the packing densities of amino acid residues in subunit interfaces and in subunit interiors are the same, both resembling amino acid crystals. This suggests that the energetics for the folding of polypeptide chains into globular subunits and for the assembly of subunits into oligomeric structures are similar. Thus, self-associating systems make simple models for folding energetics which feature well-defined initial and final states and which involve relatively few interactions. The conformational entropy of folding transitions is avoided. Con A, which exhibits only two molecular weight species related by a single equilibrium constant, provides the simplist possible model.

It is necessary to measure equilibrium constants over a wide temperature range to obtain accurate thermodynamic parameters. For typical, pH-dependent self-association reactions it is also necessary to analyze the pH dependence in order to subtract out the thermodynamics of association-linked ionization reactions. Because this approach has not often been followed for proteins whose three-dimensional structure is known, there is very little available data that can be applied to deduce the roles of the various types of interactions involved in maintaining oligomeric structures. An earlier study of the Con A self-polymerization ignored the effect of association-linked ionization reactions (Huet & Clavarie, 1978).

In the present work, we have studied the Con A self-association from 5 to 35 °C and between pH 5.5 and pH 7.5 by using the high-speed sedimentation equilibrium technique and computer programs developed in this laboratory (Teller, 1973). The pH dependence was analyzed by the Wyman linked function theory to separate the self-association and association-linked ionization reactions. Truncated van't Hoff equations were used to express the temperature dependence of the reactions. The group responsible for the pH dependence was identified from the thermodynamic parameters deduced for the association-linked ionization reaction.

Experimental Procedures

Chemicals. Concanavalin A, prepared from jack bean meal by using acetic acid as per Olson & Liener (1967) and lyophilized, was purchased from Sigma Chemical Co. (Type IV, lot no. 16C-7090). The Con A supplied by Sigma contains ~50% fragmented chains.[2] These were removed by precip-

† From the Department of Biochemistry, School of Medicine, University of Washington, Seattle, Washington 98195. *Received September 24, 1980.* This work was supported by National Institutes of Health Grant GM 13401.

‡ Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

[1] Abbreviations used: Con A, concanavalin A; rms, root mean square; $NaDodSO_4$, sodium dodecyl sulfate.

[2] About 50% of concanavalin A subunits in commercial preparations are hydrolyzed between residues 118 and 119. We term such preparations as "fragmented". However, the hydrolyzed subunits maintain their normal folded structure. In no case are materials smaller than dimer observed in sedimentation equilibrium of this Con A (see Figure 2).

0006-2960/81/0420-3076$01.25/0

itation in NH_4HCO_3 as described by Cunningham et al. (1972). Their procedure was modified slightly by first preparing the Con A at 10 mg/mL in 0.005 M sodium acetate, pH 2.3, and then dialyzing this solution against 1% NH_4HCO_3, pH 7.90, at 37 °C for 12 h. After removal of the pellet, the supernatant was filtered through a 22-μm millipore filter. When dialyzed exhaustively into the phosphate buffer described below (pH 6.5), these solutions were exceptionally stable and could be stored for up to 12 weeks at 4 °C without any deterioration detectable by ultracentrifuge analysis.

Solutions of Con A were prepared by dialysis at 20 °C in 0.05 M sodium phosphate buffer containing 0.2 mM $CaCl_2$ and $MnCl_2$, 0.1 mM NaN_3, and enough NaCl to bring the total ionic strength to 0.5 M at the desired pH. The final protein concentration was estimated spectrophotometrically at 280 nm by using an extinction coefficient, $E_{cm}^{\%} = 11.4$ (Agrawal & Goldstein, 1968). All buffer components were reagent grade products of J. Baker Chemical Co. or Mallinckrodt Chemical Works.

Analytical Ultracentrifugation: High-Speed Sedimentation Equilibrium Technique. Sedimentation equilibrium experiments were performed in a Beckman Spinco Model E ultracentrifuge equipped with electronic speed control. Data were collected as Rayleigh interference patterns, photographed on Kodak II-G spectroscopic plates. A mercury arc light source, a Baird-Atomic B-9 interference filter with a 546-nm transmittance maximum, and an interference mask with the slit width enlarged to 0.014 in. were used to produce the fringes. Temperature was controlled by an RTIC temperature control unit. At least 12 fringes were routinely resolved. Thus the effective concentration range measured was 0.1–3 mg/mL. For runs at temperatures >25 °C, the chamber was lined with mirrored stainless steel to avoid excessive use of the heater (Aune et al., 1971).

Centrifuge experiments were performed at 18 000–22 000 rpm in Yphantis-style six-channel charcoal-filled epoxy centerpieces with matched sapphire windows. Solution channels were filled to a 3-mm column height with 0.13 mL of Con A solution, which had been dialyzed against the appropriate buffer. A 3:1 dilution series (0.5, 1.0, and 1.5 mg/mL) of Con A was used with dialyzate placed in the solvent channels. Photographs were taken at least 4 h after the time required to reach equilibrium (12–14 h), calculated according to Teller (1973). Sedimentation equilibrium tracked the temperature closely in runs in which the temperature was changed after equilibrium was attained, implying that chemical equilibrium is rapid relative to the time scale of the experiment. Photographs were taken at least 1 h after the new temperature was reached. For these runs, the pH of the buffer was calculated at each temperature other than 20 °C by using the data from Bates & Acree (1943). Base line photographs were taken at 3200 rpm after shaking the assembled cell in a test-tube vortex mixer.

Method of Analysis. Equilibrium and base line photographs were read on a modified automated Nikon microdensitometer described by De Rosier et al. (1972) and interfaced to a digital equipment PDP-12 computer. The system uses Fourier transform analysis to determine the displacement from the position of the zeroth-order fringe. Crude base line and equilibrium data were smoothed, and point-by-point number-, weight-, and z-average molecular weight moments were calculated as described by Teller (1973). Point-by-point association constants for the reaction $2D \rightleftarrows T$ were calculated from each molecular weight moment according to expressions given by Hoagland & Teller (1969), and a weighted-average value

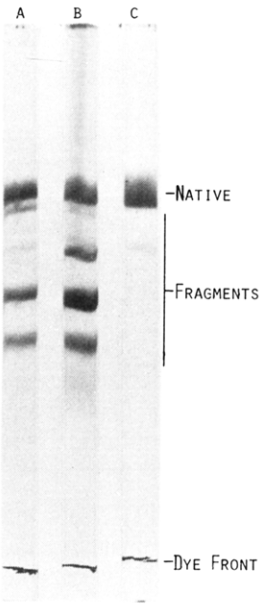


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of Con A. These are 15% gels, electrophoresed for 4 h at 4 mA/gel and 20 °C. The gels are (a) commercial Sigma Type IV Con A, (b) the pellet after precipitation of Con A in 1% ammonium bicarbonate, and (c) the ammonium bicarbonate supernatant fraction.

of the association constant (k_2) was calculated for each experiment. The root-mean-square deviation of the predicted vs. the observed weight-average molecular weights was used to assess how well the model described the data. For these calculations, the dimer molecular weight was always taken to be 51 000, the analytical dimer molecular weight (Becker et al., 1975). The data were always best described by a dimer molecular weight, $M_2 = 51\,000 \pm 1500$. The partial specific volume was calculated from data given in Cohn & Edsall (1943) and the amino acid composition (Cunningham et al., 1975). This gave $\bar{v} = 0.731$ mL·g^{-1} at 20 °C in agreement with Sumner's measurement (Sumner et al., 1938). The temperature dependence of $\bar{v}$ was taken to be $d\bar{v}/dT = 3.7 \times 10^{-4}$ mL·g^{-1}·deg^{-1} (Bull, 1976). A refractive index increment of 4 fringes·mg^{-1}·mL was assumed.

Results

The result of the purification of native intact Con A from the Sigma product is shown in Figure 1. In the presence of 1% $NaDodSO_4$ on polyacrylamide gels, the Sigma Type IV Con A shows the pattern described by Wang et al. (1971) as being typical for Con A purified by crystallization or as per Olson & Liener (1967). The Sigma product appears to contain about equal amounts of native polypeptide chains and hydrolyzed fragments. The NH_4HCO_3-purified fraction is substantially free of fragments.

The reversibility and stoichiometry of the self-association reaction were tested by high-speed sedimentation equilibrium of a single Con A sample at pH 6.70 which was run to equilibrium at 25 °C and again at 20 °C. The pH was adjusted by dialysis to 7.30, and the experiment was repeated. Typical molecular weight average distributions (Figure 2) are independent of the initial protein concentration as shown by the superimposability of the curves in the figure, indicating a chemically reversible equilibrium (Teller, 1973). There is a smooth transition in the molecular weight moments between the analytical dimer (51 000) and tetramer (102 000) molecular weights as a function of concentration. At each experimental condition, the two-component plot of molecular weight aver-

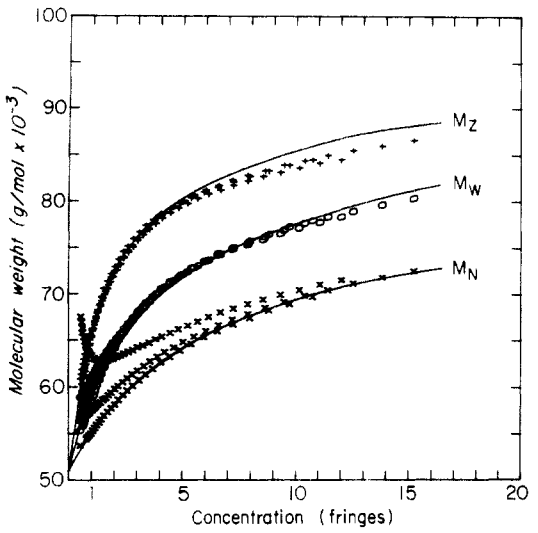


FIGURE 2: Molecular weight distribution of commercial Con A obtained by sedimentation equilibrium. Data presented are computer-generated plots of molecular weight vs. concentration (fringes) from analysis of a Rayleigh plate. Initial loading concentrations of approximately 0.5, 1.0, and 1.5 mg/mL were centrifuged for 17 h at 22 000 rpm and 20 °C in 0.14 M sodium phosphate, pH 6.70, and 0.2 mM $CaCl_2$ and $ZnCl_2$. The solid curves represent the best fit to the data with a dimer molecular weight, M_2 = 51 000, calculated according to Teller (1973). This gave ln k_2 = 10.06 ± 0.05 rms. M_n, M_w, and M_z represent the number-average molecular weight, the weight-average molecular weight, and the z-average molecular weight.

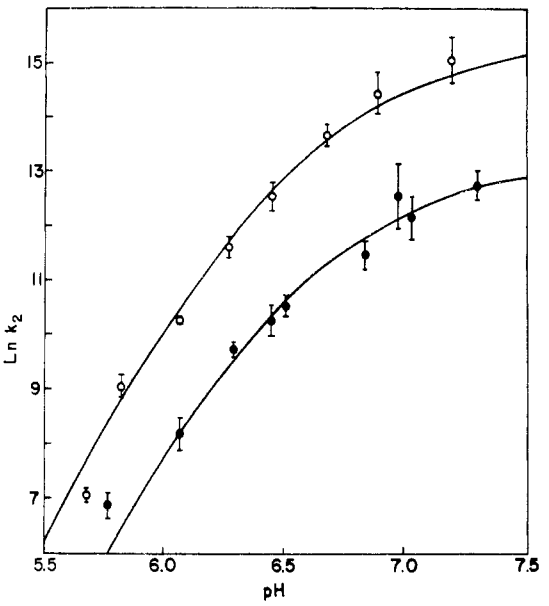


FIGURE 3: Natural logarithm of observed dimer to tetramer self-association constant for Con A vs. pH at 20 °C. The points are calculated from sedimentation equilibrium data as described in Figure 2. The phosphate buffer described under Experimental Procedures was used. (○) Ammonium bicarbonate purified, intact Con A; (●) commercial (Sigma) Con A. Error bars represent rms deviation. The solid curves are the best fits of eq 1 to the data. For intact Con A (○) this gave pK_{D,H^+} = 6.55 and for commercial Con A (●), pK_{D,H^+} = 6.47.

ages developed simultaneously by Horbett (Teller et al., 1969) and Roark (Roark & Yphantis, 1969) did not indicate the presence of monomer, trimer, or polymer species larger than the tetramer. Dyson analysis of the data (Van Holde et al., 1969; Teller, 1973) where number, weight, and z averages were used simultaneously to determine equilibrium constants found zero or negative constants for the formation of polymers larger than tetramers. The data were best described by a model that included only dimer and tetramer species in a single, rapid, reversible equilibrium, confirming the hypothesis that such an equilibrium exists under these conditions (Huet, 1975). Attempts were made to include the effect of nonideality in the Dyson calculations. These invariably produced negligibly small or negative second virial coefficients. For the concentration range of the sedimentation equilibrium experiments, i.e., <4 mg/mL, the activity coefficients of all protein species were taken to be unity. Equilibrium constants calculated as described above showed that the equilibrium is both pH and temperature dependent. Molecular weight data were well described by a chemically ideal dimer–tetramer equilibrium between 5 and 35 °C and from pH 5.5 to pH 7.5.

The observed pH dependence implies that ionizable groups govern the self-association reaction. The reaction with purified intact Con A was titrated at 20 °C in phosphate buffer to give the upper set of points in Figure 3. Molecular weight data such as that in Figure 4 confirms the reversibility of the reaction and the correctness of the dimer–tetramer model over this entire range of pH. Below pH 5.5 Con A appears to be dimeric with the self-association increasing to a limiting value at pH ~7.5. The data were analyzed according to the Wyman theory of linked functions (Wyman, 1964). The maximum slope of the titration curve is a lower limit to the number, n, of association-linked proton binding sites. The data in Figure 3 indicates n = 4, or one site per subunit of the tetramer. The apparent pK, ~6.5, is consistent with a histidine side chain. The data show no inflection or evidence of a limiting value of ln k_2(obsd) at low pH to indicate the binding of protons

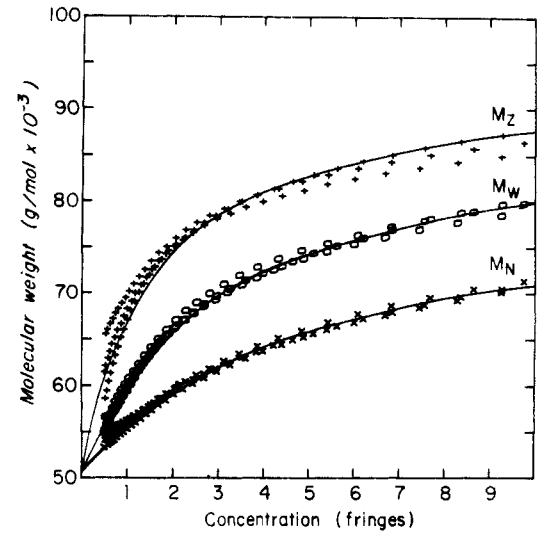
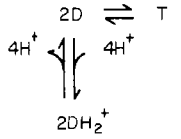


FIGURE 4: Molecular weight distribution of ammonium bicarbonate purified, intact Con A obtained by sedimentation equilibrium. Initial loading concentrations of approximately 0.5, 1.0, and 1.5 mg/mL were centrifuged for 17 h at 20 000 rpm and 20 °C in the phosphate buffer described under Experimental Procedures, pH 6.09. The solid curves represent the best fit to the data with M_2 = 51 000, calculated according to Teller (1973). This gave ln k_2 = 10.30 ± 0.06 rms.

to the linked group in the tetramer. The model that fits these data is given by

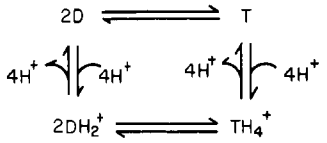


where T represents the tetramer and D and DH_2^+ are unprotonated and protonated dimers. This scheme is described by

$$\ln k_2(\text{obsd}) = \ln k_{2,\nu_{H^+}=0} - 4 \ln (1 + a_{H^+}/K_{D,H^+}) \quad (1)$$

where k_2(obsd), $k_{2,\nu_{H^+}=0}$, and K_{D,H^+} refer respectively to the observed dimerization constant at any pH, the dimerization constant at 0 proton binding, and the dissociation constant for protons from the dimer. Least-squares solutions of eq 1 predict the solid curves in Figure 3, where the lower set of points was generated by the Sigma Con A. For Sigma Con A, values of $\ln k_2$(obsd) were observed to be uniformly lower than for the intact subunit fraction. A model to explain this phenomenon in detail will appear elsewhere (unpublished experiments). Our conclusion is that dimers that contain one or two fragmented subunits comprise a fraction of incompetent dimeric species (McKenzie & Sawyer, 1973).

A second model that was considered is



This scheme is described by

$$\ln k_2(\text{obsd}) = \ln k_{2,\nu_{H^+}=0} + 4 \ln [(1 + a_{H^+}/K_{T,H^+})/(1 + a_{H^+}/K_{D,H^+})] \quad (2)$$

Using least-squares techniques, we found no reasonable solution to eq 2. With three adjustable parameters, no positive value for K_{T,H^+} could be found. When K_{D,H^+} was fixed, the $DH_2^+ \rightleftarrows TH_4^+$ equilibrium constant was unreasonably small. Thus, the data do not support the hypothesis that protonated dimer, DH_2^+, is capable of forming the tetramer, TH_4^+.

For determination of thermodynamic parameters for the self-association and association-linked proton dissociation reactions, the temperature dependence of the titration curve for purified intact Con A in Figure 3 was examined. A temperature-dependent form of eq 1 was written, with each equilibrium constant expanded as a three-term van't Hoff series to give

$$\ln k_2(\text{obsd}) = a_1 + b_1(1/T) + c_1 \ln T - 4 \ln (1 + a_{H^+} \exp[-[a_2 + b_2(1/T) + c_2 \ln T]]) \quad (3)$$

The high-speed sedimentation equilibrium technique relies strongly on the low concentration data for high values of $\ln k_2$(obsd) (>15 in this case). The accuracy of these data is limited by rotor vibration which causes convection and systematically low apparent equilibrium constants. For low values of $\ln k_2$(obsd) random error may lead to molecular weight averages of <51 000 which are necessarily ignored when calculating $\ln k_2$(obsd), leading to systematically high values. For the purified intact chain fraction of concanavalin A, the pH and temperature range over which $\ln k_2$(obsd) could be measured independent of these effects was not sufficient to be fit directly by eq 3. It was possible to reconstruct what the entire pH- and temperature-dependent surface of $\ln k_2$(obsd) would be for purified intact Con A from data collected for the Sigma Type IV preparation, with its proportion of fragmented subunits.

For the Sigma Con A, $\ln k_2$(obsd) was observable over the entire range from 5 to 35 °C between pH 5.5 and pH 7.5, sufficient to analyze directly with eq 3. Thus, the coefficients a_2, b_2, and c_2 of eq 3 could be determined for this material. Further, the titration curves in Figure 3 are parallel relative to the pH axis, within the limits of experimental uncertainty. Application of eq 1 to the solid curves of Figure 3 gave pK_D = 6.55 for the purified intact subunit Con A and 6.47 for the Sigma material. The titration of the purified Con A could be fit to eq 1 with the value pK_D = 6.47 entered as an invariant parameter to give the 20 °C curve in Figure 6. Since this is not the least-squares solution, the rms[1] deviation from the observed points does increase a small amount. However, this model is still an excellent description of the data for the purified intact Con A. Titration curves for the two preparations were observed to be parallel at other temperatures as well. Inspection of eq 3 shows that this is equivalent to the statement that the coefficients a_2, b_2, and c_2 for the Sigma Con A and a_2, b_2, and c_2 for the intact Con A are equal. On this basis, data for Sigma Con A were used to deduce thermodynamic parameters for the association-linked proton dissociation reaction. The pH-dependent term so determined (last term on the right of eq 3) was subtracted from both the right-hand side of eq 3 and from the values $\ln k_2$(obsd) for the purified intact Con A to determine thermodynamic parameters for the self-association of the intact material.

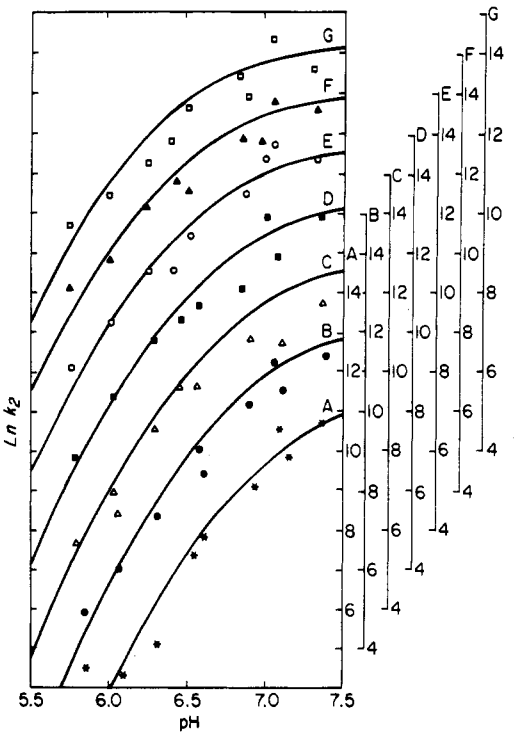


FIGURE 5: Natural logarithm of observed dimer to tetramer self-association constant vs. pH for commercial (Sigma) Con A at several temperatures. The points were calculated from sedimentation equilibrium data as described in Figure 2. The solid curves represent the best fit of eq 3 to the experimental points, which gave the values listed in Table I. Curves are offset for clarity. The symbols are as follows: curve A (*), 5 °C; curve B (●), 10 °C; curve C (▲), 15 °C; curve D (■), 20 °C; curve E (○), 25 °C; curve F (▲), 30 °C; curve G (□), 35 °C.

Using Sigma Con A, $\ln k_2$(obsd) was determined as a function of temperature and pH in a series of experiments in which a single sample of protein was centrifuged to equilibrium at 35 °C, and then the temperature was dropped in 5 °C increments to 5 °C (Figure 5). When the model given by eq 3 was fit to these 69 data points, a negligibly small value for the coefficient c_2 was found. This coefficient gives ΔC_p for the proton dissociation reaction. A five adjustable parameter version of eq 3 with c_2 = 0 fixed was also used to fit the data. An error analysis (Hildebrand, 1956) showed the sixth parameter to be unjustified. The coefficients determined for the Sigma Con A association behavior are given in Table I. At 20 °C, the apparent equilibrium constant for the self-association of Sigma Con A is 5.6×10^5 M^{-1} at the condition ν_{H^+} = 0. The reaction is governed by a single group

Table I: Coefficients[a] to Equation 3 Determined for Concanavalin A

prepn	Sigma[b]	purified,[c] intact
a_1	2.1450×10^3	2.7998×10^3
b_1	-9.9960×10^4	-1.2836×10^5
c_1	-3.1524×10^2	-4.1305×10^2
a_2	1.8351	1.8531
b_2	3.8227×10^3	3.8227×10^3
c_2	0	0

[a] For the Sigma Con A, the coefficients are from the least-squares solution of eq 3. For the purified, intact Con A, the coefficients a_1, b_1, and c_1 are from the least-squares solution of eq 4 while the coefficients a_2, b_2, and c_2 are taken from the Sigma Con A solution. [b] Sigma Type IV Con A used with no further purification. [c] Supernatent fraction of Sigma Type IV Con A after precipitation in 1% ammonium bicarbonate.

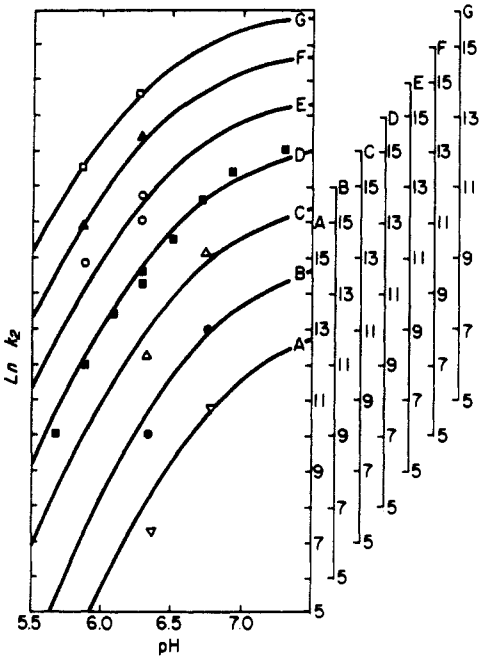


FIGURE 6: Natural logarithm of observed dimer to tetramer self-association constant vs. pH for ammonium bicarbonate purified, intact Con A at several temperatures. The solid curves represent the fit of eq 3 to the experimental points as described in the text, using values listed in Table I. Symbols are as follows: curve A (▽), 5 °C; curve B (●), 10 °C; curve C (△), 15 °C; curve D (■), 20 °C; curve E (○), 25 °C; curve F (▲), 30 °C; curve G (□), 35 °C.

on each subunit with $pK_{20} = 6.47 \pm 0.10$.

For the purified intact subunit fraction of Con A, ultracentrifuge analysis was performed as described between 20 and 35 °C from pH 5.5 to pH 6.3 and between 5 and 20 °C from pH 6.3 to pH 7.5 (Figure 6). Subtraction of the contribution of the linked proton dissociation reaction gave the calculated values for ln $k_{2,\nu_{H^+}=0}$ in Figure 7. These were analyzed by

$$\ln k_2(\text{calcd}) = a_1 + b_1(1/T) + c_1 \ln T \qquad (4)$$

to obtain the solid theoretical curve in the figure. The coefficients determined for the self-association behavior of intact subunit Con A are given in Table I. At 20 °C under these conditions, the equilibrium constant for the self-association of intact or native Con A dimers is 5.5×10^6 M^{-1}, which is a factor 10 times the apparent constant for the Con A that is contaminated by ~50% fragmented chains.[2]

Since the primary objective of this work was to provide an energetic description of the concanavalin A self-association that could be compared to the known subunit–subunit contact

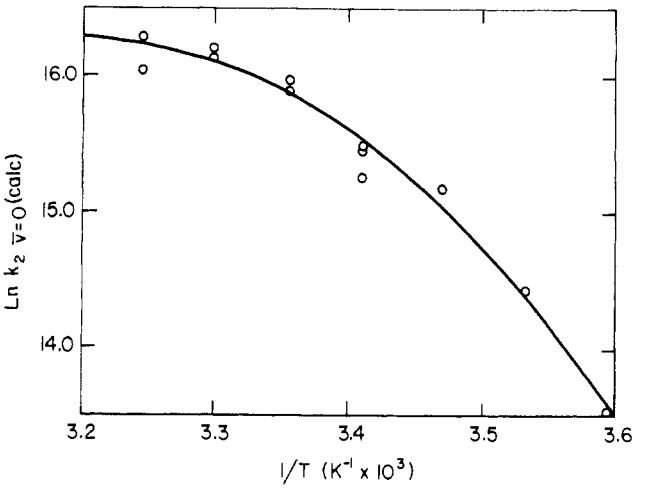


FIGURE 7: van't Hoff plot of equilibrium constants for dimer to tetramer association of ammonium bicarbonate purified, intact Con A at the condition $\nu_{H^+} = 0$ at the linked proton site. The abscissa values were calculated from experimental points in Figure 6 by using eq 1 and pK_{D,H^+} values calculated from the vaues for a_2, b_2, and c_2 in the second column of Table I, as described in the text. The solid curve is the best fit of eq 4 to the points which gave the values of a_1, b_1, and c_1 the third column of in Table I.

Table II: Standard Thermodynamic Parameters[a] of the Dimer ⇆ Tetramer Equilibrium of Con A in Solution at $\nu = 0$ Condition

t (°C)	ΔG (kcal·mol^{-1})	ΔH (kcal·mol^{-1})	ΔS (cal·mol^{-1}·deg^{-1})	ΔC_p (cal·mol^{-1}·deg^{-1})
15	−8.59	18.6	94.3	−821
20	−9.03	14.5	80.1	−821
25	−9.39	10.4	66.3	−821

[a] Calculated from the coefficients in the last column of Table I for purified, intact Con A. [b] ΔG has an rms deviation from the observed points of 0.12 kcal·mol^{-1} or <1.5%. ΔH and ΔS are determined to better than ±20% and ΔC_p to ±45%.

Table III: Standard Thermodynamic Parameters for Proton Dissociation Reactions of Con A and Imidazole Derivatives at 25 °C

compound	ΔG (kcal·mol^{-1})	ΔH (kcal·mol^{-1})	ΔS (cal·mol^{-1}·deg^{-1})	ΔC_p (cal·mol^{-1}·deg^{-1})
Con A[a]	8.69	7.60	−3.68	0
imidazolium chloride[b]	9.54	8.76	−2.62	0.45
histidine (pK_2)[c]	8.18	6.90	−4	

[a] This refers to the site linked to the self-association reaction. The parameters are calculated from the coefficients in Table I. [b] Data are taken from Datta & Grysbowski (1966). [c] Data for the side-chain proton dissociation reaction are taken from Cohn & Edsall (1943).

areas to attempt to assess the roles of the various interactions in the reaction, the standard thermodynamic parameters $\Delta G°$, $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ for the association ($\nu_{H^+} = 0$) and for the linked ionization were determined. Their values are listed at several temperatures in Tables II and III. The precision of $\Delta G°$ for the observed association reaction is calculated from the rms deviation (equal to 0.208) of the values predicted by the coefficients in Table I from the values for ln k_2(obsd) to be 0.12 kcal·mol^{-1}. The precisions of $\Delta H°$ and $\Delta S°$ for the reaction are estimated by propagation of errors to be 22% and 18% at 20 °C. Since $\Delta C_p°$ is taken from the second derivative of the observed quantity as a function of temperature, it is

poorly determined. The precision is estimated to be ±45%.

Discussion

The data presented verify that the self-association of concanavalin A is strictly a dimer to tetramer transition over the pH range 5.5–7.5. This agrees generally with the conclusion of an earlier study (Huet & Clavarie, 1978), whose authors were uninterested in the pH dependence of the reaction and did not appreciate the implications of the presence of a proportion of fragmented[2] subunits in commercial Con A preparations (Wang et al., 1971). In the case of our high-grade commerical preparation of Con A, we find that about half the subunits are fragmented and that apparent association constants are 9–12-fold smaller than for a preparation from which fragmented subunits are removed. This implies that dimers containing fragmented subunits are less competent to form tetramers than are intact dimers. Most fragmented subunits are hydrolyzed at the same single point (Wang et al., 1975), yielding a single species of fragmented subunit. This would produce three possible dimer types and six different self-association equilibria.

We find that the dimer–tetramer model holds to 35 °C and, in the presence of α-methyl mannosides or glucosides, to 37 °C (data not shown). Huet & Clavarie (1978), who collected band sedimentation data under conditions remarkably similar to ours, claim that an isodesmic association occurs above 23 °C. We have found that at low ionic strength (0.15–0.2 M) aggregates of Con A form irreversibly. This explains the earlier result since the Con A samples were prepared in phosphate-buffered saline at the start of the sedimentation experiments. Aggregates probably affect their data at lower temperatures as well, explaining why their reported equilibrium constants at pH 7.2 for a Miles Yeda Con A preparation are generally larger than what we calculate for our Sigma Con A. Their 23 °C value is close to what we calculate for our intact subunit fraction, implying that the effects of higher aggregates and incompetent dimers may have fortuitously cancelled each other. At 4 °C, where fewer aggregates form, the constant reported by Huet & Clavarie (1978) is closer to the value for our commercial Con A.

From the values of the parameters listed in Table III, it is clear that the ionization state of a histidine side chain on each subunit governs the dimer–tetramer transition of concanavalin A. From the inability of the model given by eq 2 to describe the data, we conclude that this histidine is in the dimer–dimer interface, is inaccessible to solvent in the tetramer, and would form a highly unfavorable electrostatic interaction in the tetramer, if protonated. Using the coordinates and subunit numbering scheme of Reeke et al. (1975) and accessible surface area calculations similar to those described by Shrake & Rupley (1973), we have made atom by atom diagonal plots (Phillips, 1970; Nishikawa & Ooi, 1972; Nishikawa et al., 1972; Rossman & Liljas, 1974) for all atoms that change accessible surface area in forming either the subunit I–subunit II or subunit I–subunit III contacts in an effort to identify which histidine is involved. We concur with Reeke et al. (1975) that it is the subunit I–subunit II contact (and the III–IV also) that defines the solution dimer. These subunits have extensive contact between backbone atoms in a region of regular secondary structure (Reeke et al., 1975; Hardman & Ainsworth, 1972) while the I–III (and II–IV) interface has fewer tight contacts, has tight contacts only between side-chain atoms, and shows no regular structure. We have identified two histidine side chains, at residue positions 51 and 121, which are almost completely accessible to solvent in the dimer and are completely buried in the tetramer.

One ring nitrogen of histidine-51 of subunit I is within 4 Å of the side chain of Lys-116 of subunit II, which is in turn part of a charge–charge system involving also Lys-114 of subunit II and Glu-192 of subunit I. Proton binding by histidine-51 would result in a high density of positive charge balanced by a lone negative charge. On the other hand, the four histidine-121 residues in the tetramer are near the point of 222 symmetry in a region of local crystal disorder (Reeke et al., 1975) and are <10 Å from each other. The possibility that mutual charge repulsion between these side chains causes the tetramer to dissociate cannot be completely dismissed. Free energy calculations due to the electrostatic potential (Tanford, 1961) were performed on the Con A tetramer and dimers under a variety of conditions of protonation of histidine-51 and -121. It proved impossible to distinguish between these residues on the basis of the resultant, predicted free energy differences. Since the data could not be fit by a model involving eight linked protons and do not justify more than a single proton dissociation constant, it cannot be the case that both histidines are involved or that it is sometimes one and sometimes the other.

The enthalpies and entropies reported by Huet & Clavarie (1978) are in general agreement with our values (Table II). This is because the effect from the proton dissociation reaction is small at pH 7.2. Their values are more positive than ours, but their method of analysis is less accurate and, contrary to what is reported, the precision is only half as good, based on the reported precision of ΔG. In addition, the entropy and enthalpy reported between 15 and 23 °C in their Table IV are inconsistent with the free energy reported at either temperature.

The values of the thermodynamic parameters listed in Table II show that the self-association reaction is entropically driven. this and the large negative heat capacity aree consistent with the usual notion of hydrophobic interactions (Scheraga, 1963). This is in agreement with the crystallographic studies of Reeke et al. (1975) which indicate that the region of dimer–dimer contact is a large, hydrophobic, antiparallel pleated sheet structure.

More interesting is the large positive enthalpy of dimerization. As a variety of authors [e.g., most recently Finney et al. (1980)] have pointed out, the enthalpy for a process such as a protein self-association is the relatively small sum of several large competing contributions. Therefore, it is dangerous to attempt to assign an observed enthalpy to any one effect. Nevertheless, the enthalpy for the Con A dimerization is an unexpectedly large, positive number (Ross & Subramanian, 1980), and it is enticing to make an assignment. Huet & Clavarie (1978) explain that the interdimer hydrogen bonds and salt links in the tetramer must be less favorable than the dimer–solvent interactions they replace. However, the number of interdimer salt links and hydrogen bonds is small relative to other proteins when compared as a function of association contact area, and, since our analysis of the interface reveals nothing particularly unusual about them, we do not feel that this is the best explanation for an anomalously large heat of association. We prefer to suggest the involvement of the region of the tetramer surrounding the 222 symmetry point, including primarily Gln-122, which shows local disorder in the crystalline tetramer (Reeke et al., 1975). The hypothesis is that strain energy or steric hindrance makes a significant contribution to the enthalpy.

On the basis of correlations between solvent cavity area and the transfer free energy of amino acid side chains from water to ethanol or dioxane (Nozaki & Tanford, 1971; Herman,

Table IV: Contributions to Unitary Entropy of Concanavalin A Dimer–Tetramer Transition at 25 °C

source	ΔS_u (cal·deg^{-1})	ΔC_p (cal·mol^{-1}·deg^{-1})	ΔG_u (kcal)
obsd[a]	74.2	−821	−22.1
hydrophobic[b]	185.8	−714.6	−55.4
vibrational[b]	−111.6	−106.2	33.2
hydrophobic[c]	185		−55.3

[a] The observed unitary entropy was calculated from $\Delta S_u = \Delta S^\circ - R \ln (1/55.51)$. [b] These refer to the contributions predicted by the analysis of Sturtevant (1977). [c] This derives from the loss of accessible surface area (see text) and Chothia's (Chothia, 1974) estimate of the free energy of a hydrophobic interaction.

1972), Chothia (1974) has suggested that 24 cal/Å^2 is a good estimate for the free energy of a hydrophobic interaction. According to Kauzman (1959) this is an entropic effect. We calculate the accessible surface area lost to be 2305 Å^2 for the Con A dimerization. From Chothia's estimate, the expected contribution to the entropy is 185 eu or −55.3 kcal·mol^{-1} in free energy at 25 °C. This is 3 times the observed ΔS, indicating that there are nonhydrophobic contributions to the entropy. Sturtevant (1977) has suggested that changes in low-energy vibrational states contribute to the entropy for processes involving proteins. His analysis was developed from a consideration of cases of ligand binding and folding transitions but has not yet been applied to cases of self-association reactions. To make the calculation, we assume that there is no conformational contribution to the entropy. Considering the local crystal disorder of the tetramer around the 222 point, this is not strictly correct, but there is evidence (Pflumm et al., 1971; McCubbin et al., 1971; Kay, 1970) to suggest that it is not a bad assumption overall. Also, the analysis is relatively insensitive to the value of ΔS(conformational). Applying the theory gives the values for the contributions listed in Table IV. What is remarkable is the consistency between the calculated ΔS(hydrophobic) and that expected on the basis of Chothia's estimate. The difference accounts for ~8 Å^2 of surface area. This result also appears to be true for the case of the low-pH dimerization of chymotrypsin (D. C. Teller, unpublished experiments).

In conclusion, we have shown that Con A is a stable, reversible dimer–tetramer system in the neutral pH range over a large range of temperature and that the reaction is governed by proton binding to a single histidine residue on each subunit. The presence of fragmented chains in commercial Con A preparations has a large effect on the phenomemological association constant, greatly increasing the fraction of dimer present under all conditions. This is important because of the implication that large amounts of dimer are present under conditions where cell agglutination and other biological effects are observed. Using data for the salt dependence of the reaction (Senear & Teller, 1981), we calculate that the Sigma Con A is 50% dimer at 100 μg/mL and 35 °C in phosphate-buffered saline. We hope to be able to use the thermodynamic data presented along with data for other proteins, e.g., hemoglobin (Chu & Ackers, 1981) and chymotrypsin (D. C. Teller, unpublished experiments), to deduce the roles of hydrophobic, hydrogen-bond, and electrostatic interactions in protein self-assembly processes.

References

Agrawal, B. B. L., & Goldstein, I. J. (1968) *Arch. Biochem. Biophys. 124*, 218–229.

Aune, K. C., Goldsmith, L. C., & Timasheff, S. M. (1971) *Biochemistry 10*, 1617–1622.

Bates, R. G., & Acree, S. F. (1943) *J. Res. Natl. Bur. Stand. 30*, 129–155.

Becker, J. W., Reeke, G. N., Jr., Wang, J. L., Cunningham, B. A., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1513–1524.

Bull, H. B. (1976) in *Handbook of Biochemistry and Molecular Biology, Proteins* (Fasman, G. D., Ed.) Vol. 3, pp 595, CRC Press, Cleveland, OH.

Chothia, C. (1974) *Nature (London) 248*, 338–339.

Chothia, C., & Janin, J. (1976) *Nature (London) 256*, 705–708.

Chu, A. H., & Ackers, G. K. (1981) *J. Biol. Chem. 256*, 1199–1205.

Cohn, E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides*, pp 370–381, Reinhold, New York.

Cunningham, B. A., Wang, J. L., Pflumm, M. N., & Edelman, G. M. (1972) *Biochemistry 11*, 3233–3239.

Cunningham, B. A., Wang, J. L., Waxdall, M. J., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1502–1512.

Datta, S. P., & Grzybowski, A. K. (1966) *J. Chem. Soc. B*, 136–140.

De Rosier, D. J., Munk, P., & Cox, D. J. (1972) *Anal. Biochem. 50*, 130–153.

Finney, J. L., Gellatly, B. J., Golton, J. C., & Goodfellow, J. (1980) *Biophys. J. 32*, 17–30.

Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., & Edelman, G. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1012–1016.

Hardman, K. D., & Ainsworth, C. F. (1972) *Biochemistry 11*, 4910–4919.

Herman, R. B. (1972) *J. Chem. Phys. 76*, 2754–2759.

Hildebrand, F. B. (1956) in *Introduction to Numerical Analysis*, pp 268, McGraw-Hill, New York.

Hoagland, V. D., Jr., & Teller, D. C. (1969) *Biochemistry 8*, 594–602.

Huet, M. (1975) *Eur. J. Biochem. 59*, 627–632.

Huet, M., & Clavarie, J. M. (1978) *Biochemistry 17*, 236–241.

Kalb, A. J., & Lustig, A. (1968) *Biochim. Biophys. Acta 168*, 366–367.

Kauzman, W. (1959) *Adv. Protein Chem. 14*, 1–63.

Kay, C. M. (1970) *FEBS Lett. 9*, 78–80.

McCubbin, W. D., Oikawa, K., & Kay, C. M. (1971) *Biochem. Biophys. Res. Commun. 43*, 666–674.

McKenzie, G. H., & Sawyer, W. H. (1973) *J. Biol. Chem. 248*, 549–556.

McKenzie, G. H., Sawyer, W. H., & Nichol, L. W. (1972) *Biochim. Biophys. Acta 263*, 283–293.

Nishikawa, K., & Ooi, T. (1972) *J. Phys. Soc. Jpn. 32*, 1331–1337.

Nishikawa, K., Ooi, T., Isogai, Y., & Saito, N. (1972) *J. Phys. Soc. Jpn. 32*, 1338–1347.

Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.

Olson, M. O. J., & Liener, I. E. (1967) *Biochemistry 6*, 105.

Pflumm, M. N., Wang, J. L., & Edelman, G. M. (1971) *J. Biol. Chem. 246*, 4369–4375.

Phillips, D. C. (1970) in *British Biochemistry Past and Present* (Goodwin, T. W., Ed.) pp 11–28, Academic Press, London.

Reeke, G. N., Jr., Becker, J. W., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1525–1547.

Roark, D. E., & Yphantis, D. A. (1969) *Ann. N.Y. Acad. Sci. 164*, 245–278.

Ross, P. D., & Subramanian, S. (1980) *Biophys. J. 32*, 79–81.

Rossman, N. G., & Liljas, A. (1974) *J. Mol. Biol. 85*, 177–181.

Scheraga, H. A. (1963) *Proteins (2nd Ed.) 1*, 477–594.
Senear, D. F., & Teller D. C. (1981) *Biochemistry* (following paper in this issue).
Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351–371.
Sturevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Sumner, J. B., Gralèn, N., & Eriksson-Quensel, I. B. (1938) *J. Biol. Chem. 125*, 45–48.
Tanford, C. (1961) *Physical Chemistry of Macromolecules* p 484, Wiley, New York.
Teller, D. C. (1973) *Methods Enzymol. 27*, 346–441.
Teller, D. C., Horbett, T. A., Richards, E. G., & Schachman, H. K. (1969) *Ann. N.Y. Acad. Sci. 164*, 66–101.
Van Holde, K. E., Rossetti, G. P., & Dyson, R. D. (1969) *Ann. N.Y. Acad. Sci. 164*, 279–293.
Wang, J. L., Cunningham, B. A., & Edelman, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1130–1134.
Wang, J. L., Cunningham, B. A., Waxdall, M. J., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1490–1502.
Wyman, J., Jr. (1964) *Adv. Protein Chem. 19*, 224–394.
Yasaka, T., & Kambara, T. (1979) *Biochim. Biophys. Acta 585*, 229–239.

Effects of Saccharide and Salt Binding on Dimer–Tetramer Equilibrium of Concanavalin A†

Donald F. Senear‡ and David C. Teller*

ABSTRACT: The effect of the binding of saccharide ligands on the reversible dimer–tetramer equilibrium of concanavalin A was studied by the high-speed sedimentation equilibrium technique. Both commercial and highly purified fragment-free concanavalin A preparations were used. In the case of the fragment-free preparation, there was no effect of the binding of α-methyl mannoside or α-methyl glucoside at 35 °C and at a variety of conditions of pH and ionic strength. This implies no difference in ligand binding activity between dimeric and tetrameric Con A, in contrast to an earlier report [McKenzie, G. H., & Sawyer, W. H. (1973) *J. Biol. Chem. 248*, 549–556]. There was a profound effect in the case of the commercial preparation. Dimers that contain hydrolyzed subunits appear to be incompetent to self-associate in the presence of α-methyl mannoside or α-methyl glycoside, while α-methyl galactoside, which does not bind to Con A, had no effect. The effects of very high concentrations of $CaCl_2$ (to 2.5 *m*) and NaCl (to 6.2 *m*) were also studied. The data were analyzed by an integrated form of the Tanford extension [Tanford, C. (1969) *J. Mol. Biol. 39*, 539–544] of the Wyman linked function theory, which includes preferential interactions with salt and water. The integrated form allows preferential interactions to be described as the sum of salt binding and water binding. The data were well described by salt binding alone; it was unnecessary to invoke any water binding effect. The $CaCl_2$ data did indicate that one calcium per subunit of the dimer binds to a site that is buried in the tetramer. This suggests a site on the dimer–dimer interface which is consistent with Reeke's identification of the protomers composing the solution dimer [Reeke, G. N., Jr., Becker, J. W., & Edelman, G. M. (1975) *J. Biol. Chem. 250*, 1525–1547].

Interest in concanavalin A (Con A)[1] is generally associated with its rather remarkable biological properties. However, Con A undergoes a rapid, reversible dimer–tetramer transition (Huet, 1975; Senear & Teller, 1981) which is also of interest, both as it may affect the biological activity of this lectin and as a model system to study protein folding and subunit aggregation interactions, since the structure is well-known from high-resolution crystallographic studies (Hardman & Ainsworth, 1972; Reeke et al., 1975). Our interest has been to provide as complete a thermodynamic description as possible for this transition, and for similar transitions involving other proteins of known structure, and to use the information to deduce the energetics of the noncovalent interactions involved in maintaining the folded structures of globular proteins. Secondarily, we hope to define the quaternary structure of Con A in solution under conditions relevant to its biological application.

Previously, we have shown that over wide ranges of temperature and pH, Con A consists of only dimers and tetramers (Senear & Teller, 1981) and that the large fraction of hydrolyzed subunits in commercial preparations (Kalb & Lustig, 1968; Wang et al., 1971) causes significant populations of dimeric species that associate only weakly or not at all (McKenzie & Sawyer, 1973; Senear & Teller, 1981). The Con A dimer–tetramer transition is typically pH dependent (McKenzie et al., 1972), the degree of association being governed by the ionization state of a single histidyl residue on each subunit (Senear & Teller, 1981). We now turn our attention to the effects of saccharide binding and to the effects of preferential interactions with solution components.

Any linkage between saccharide binding and the self-association of Con A would be relevant to the question of the quaternary structure of the protein when it is used as an analytical or biological tool. In the case of mitogenic stimulation of lymphocytes (Gunther et al., 1973) and in some other cases [e.g., Yasaka & Kambara (1979)], the activity of chemically modified dimeric Con A has been shown to be reduced relative to native Con A, suggesting that Con A must cross-link receptors. An earlier attempt to explore the possibility of such a linkage (McKenzie & Sawyer, 1973) suffered

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received October 13, 1980.* This work was supported by National Institutes of Health Grant GM 13401.
‡ Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

[1] Abbreviations used: Con A, concanavalin A; rms, root mean square; *I*, ionic strength; *K* is used to designate dissociation constants and *k*, for association constants.

0006-2960/81/0420-3083$01.25/0