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

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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

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[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.

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from the belief that Con A dimers and tetramers are not in equilibrium in solution. Circumstantial evidence for a linkage between the binding of α-methyl mannoside or α-methyl glucoside, the two most tightly bound simple saccharide ligands of Con A (Poretz & Goldstein, 1970), and the dimer–tetramer reaction includes the similarity of transitions in the near-ultraviolet circular dichroic spectrum of Con A induced by the addition of methyl mannoside or methyl glucoside and induced by a change in pH from 5.5 to 7.5 (McCubbin et al., 1971; Pflumm et al., 1971). Also, methyl mannoside has been reported to inhibit the time-dependent aggregation of Con A at pH 8.5 (McKenzie et al., 1972) while methyl galactoside, a related sugar which does not bind to Con A, has no effect.

A consequence of the association reaction is that solvent, buffer ions, and any low molecular weight solutes present must be released from the surface of the dimer–dimer interface. Interaction of the protein with any of these solution components would cause the dimer–tetramer equilibrium to be affected by the activity of that component in the solution. In particular, water molecules are expelled by the self-association process. This is of interest since the general view is that the hydrophobic force arises as an entropic effect due to the ordering of solvating water molecules around apolar surfaces of the protein (Scheraga, 1963). Thus, the effect of the preferential interactions of solution components on the Con A dimer–tetramer equilibrium bears on the question of the magnitude of the hydrophobic contribution to the association free energy. Because the dimer–dimer interface region is large and quite hydrophobic (Hardman & Ainsworth, 1972; Reeke et al., 1975) and because the association reaction is strongly entropically driven (Huet & Clavarie, 1978; Senear & Teller, 1981), the Con A system is an excellent system to study.

We have used the sedimentation equilibrium technique to measure the effects of methyl mannoside and methyl glucoside on the dimer–tetramer equilibrium of both commercial and purified intact subunit preparations of Con A. In addition, the effects of solution components on the dimer–tetramer equilibrium were systematically studied. Tanford (1969) has extended the linked function analysis of Wyman (1964) to include the effects of preferential interactions of solution components with a macromolecule. We have used an integrated form of Tanford's expression, which allows preferential interactions to be expressed as the sum of salt binding and water binding, to analyze the results.

Experimental Procedures

Chemicals. Concanavalin A, prepared from jack bean meal as per Olson & Liener (1967) and lyophilized, was purchased from Sigma Chemical Co. (Type IV, lot no. 16C-7090). The hydrolyzed subunits present in this preparation were precipitated as previously described (Senear & Teller, 1981). The procedure used is a minor modification of the procedure described by Cunningham et al. (1972). This material was stored for up to 12 weeks as a stock solution at 4 °C in 0.05 M sodium phosphate, pH 6.48, with 0.2 mM $CaCl_2$ and $MnCl_2$, 0.1 mM NaN_3, and 0.4 M NaCl without deterioration detectable by ultracentrifuge analysis.

Imidazole, 1-*O*-methyl α-D-mannopyranoside (methyl mannoside), 1-*O*-methyl α-D-glucopyranoside (methyl glucoside), and 1-*O*-methyl α-D-galactopyranoside (methyl galactoside) were Sigma products and were the highest grades available. Imidazole was further purified before use by filtration through activated charcoal and three recrystallizations. All other salts and buffer components were reagent grade products of either J. T. Baker Chemical Co. or Mallinckrodt Chemical Works.

Preparation of Samples for Ultracentrifuge Analysis. For saccharide titration experiments, Con A samples (1.5 mg) were dialyzed against 1.0 L of stock phosphate buffer at each concentration of either methyl mannoside or methyl glucoside. The large buffer volume ensured a molar excess of several orders of magnitude of saccharide relative to Con A saccharide binding sites, validating the assumption that the final concentration of free saccharide equaled the initial total concentration. A constant-temperature water bath controlled the dialysis temperature to ±0.01 °C of the ultracentrifuge run temperature to circumvent any change in the concentration of unbound saccharide in the sample caused by the temperature dependence of the binding constant for methyl mannoside or methyl glucoside.

Because $CaHPO_4$ is poorly soluble, $CaCl_2$ titrations were performed in 0.01 M imidazole–imidazolium chloride with 0.2 mM $MnCl_2$ and 0.1 mM NaN_3 added. the concentration of ionic species other than Ca^{2+} and Cl^- was small in these solutions, so that the solution was well approximated by the two-component $CaCl_2$–water system, for purposes of calculating the mean ionic activity and the activity of water. NaCl titrations were performed in the stock phosphate buffer described. Mean ionic activity coefficients for NaCl and $CaCl_2$ were taken from data tabulated in Robinson & Stokes (1970). The water activities of the $CaCl_2$ solutions were found by interpolation of water activity or osmotic coefficient data as appropriate for linearity of the data as a function of molality. Final protein concentrations were measured spectrophotometrically by using an extinction coefficient $E_{cm}^{\%} = 11.4$ (Agrawal & Goldstein, 1968).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed in a Beckman Spinco Model E ultracentrifuge exactly as previously described (Senear & Teller, 1981). At least 10 fringes were routinely resolved in photographs taken of Rayleigh interference patterns, giving an effective concentration range of 0.1 to at least 2.5 mg/mL. For saccharide titration experiments at 35 °C, the chamber was lined with mirrored stainless steel to avoid excessive use of the heater (Aune et al., 1971). These experiments were performed at 20 000 or 22 000 rpm. The higher solution densities of NaCl and $CaCl_2$ solutions and high apparent protein specific volumes required speeds of up to 34 000 rpm. Photographs were taken several hours after the time required to reach equilibrium, estimated according to Teller (1973) by making allowance for the unusual solution densities and apparent volumes.

Method of Analysis. Equilibrium and base line photographs were scanned automatically and a weighted-average equilibrium constant for the reaction $2D \rightleftharpoons T$ was calculated from point-by-point number-, weight-, and z-average molecular weight moments as previously described (Senear & Teller, 1981). For these calculations the dimer molecular weight was taken to be 51 000, the dimer molecular weight calculated from the amino acid sequence (Becker et al., 1975). For saccharide titrations, the partial specific volume was calculated as described by Cohn & Edsall (1943) using the amino acid composition of 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). Since preferential interactions with solution components cause protein apparent specific volumes to vary with salt concentration (Casassa & Eisenberg, 1964), $\bar{v} = 0.731$ mL·g^{-1} is not applicable at high NaCl or $CaCl_2$ concentration. Instead, the apparent specific volume (ϕ') was treated as an experimental parameter in NaCl and $CaCl_2$ titration experiments. At each concentration of NaCl and

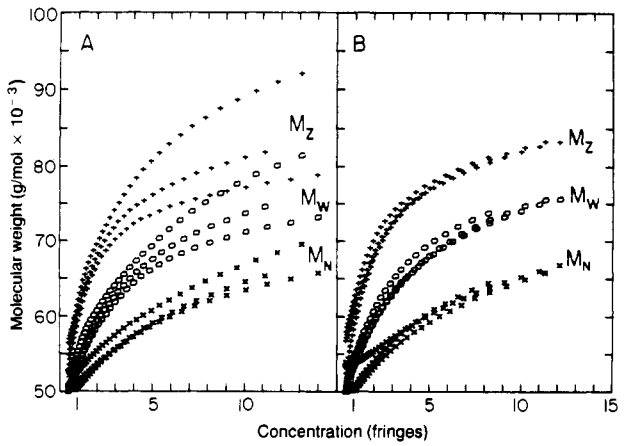

FIGURE 1: Molecular weight distributions of commercial concanavalin A obtained by sedimentation equilibrium in the presence (part A) and absence (part B) of 5.9 mM α-methyl glucoside. Data presented are computer-generated plots of molecular weight vs. concentration (fringes) from the analysis of a Rayleigh plate. Initial loading concentrations of approximately 0.5, 1.0, and 1.5 mg/mL were centrifuged for 17 h at 20 000 rpm and 20 °C in 0.05 M sodium phosphate, pH 6.47, with 0.39 M NaCl and 0.2 mM $CaCl_2$ and $MnCl_2$. M_n, M_w, and M_z represent the number-average molecular weight, the weight-average molecular weight, and the z-average molecular weight. The best fit to the data with a dimer molecular weight of M_2 = 51 000, calculated according to Teller (1973), gave ln k_2 = 9.37 ± 0.25 root mean square.

$CaCl_2$, ϕ' was calculated from the buoyancy term $(1 - \phi'\rho)$, which was adjusted until a dimer molecular weight of 51 000 best described the data. Molecular weight moments and equilibrium constants were then recalculated by using ϕ' values taken from the ϕ' vs. NaCl or $CaCl_2$ concentration calibration curves. A second method was used to estimate equilibrium constants for the $CaCl_2$ experiments. This involved fitting the c vs. r^2 data from the centrifuge directly to

$$c_r = c_{p,2}e^{-AM_2(r^2-r_p^2)} + c_{p,4}e^{-AM_4(r^2-r_p^2)}$$

where the subscripts r and p refer to the position of the experimental point and the position of an arbitrary reference point and where 2 and 4 refer to the Con A dimer and tetramer, respectively. A is given by

$$A = (1 - \phi'\rho)\omega^2/(2RT)$$

where R is the gas constant, T the absolute temperature, ρ the solution density, and ω the radial velocity. The equilibrium constant is taken to be $k_2 = c_{p,4}/c_{p,2}^2$. This method has the advantage of applying the least-squares criterion to the parameter ϕ' as well as to k_2 but has the disadvantage that since the dimer–tetramer model is assumed, there is no independent measure of its validity. In most cases, equilibrium constants estimated by the two methods agreed closely.

Results

Saccharide Binding Effects. The effect of a saturating concentration of methyl glucoside on the dimer–tetramer equilibrium of our commercial (Sigma) preparation of Con A is illustrated in Figure 1. Molecular weight average distribution patterns similar to those in Figure 1A were also observed in the presence of saturating concentrations of methyl mannoside. No effect was observed with methyl galactoside. The molecular weight moments of Figure 1A depend upon the initial concentration of protein in the centrifuge cell so that the data from the three channels of an Yphantis centerpiece do not superimpose. The observed molecular weights decrease with increasing initial protein concentration, an effect which

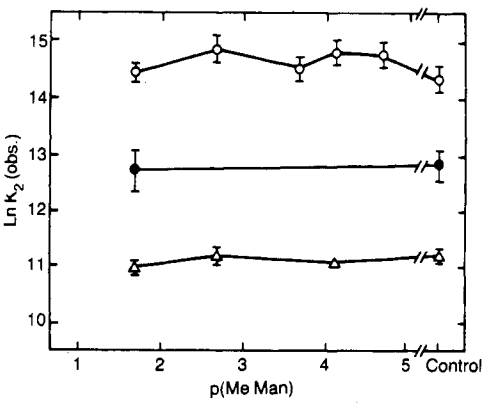

FIGURE 2: Natural log of observed equilibrium constant for dimer–tetramer association of concanavalin A measured by sedimentation equilibrium as a function of –log of α-methyl mannoside (MeMan) concentrations. The control is in the absence of methyl mannoside. The experiments were conducted at 35 °C in 0.05 M sodium phosphate and 0.39 M NaCl with 0.2 mM $MnCl_2$. Samples were centrifuged at 20 000 or 22 000 rpm for 16 h. The symbols are as follows: (○) pH 6.5, 0.2 mM $CaCl_2$; (●) pH 5.8, 2.0 mM $CaCl_2$; (△) pH 5.8, 0.2 mM $CaCl_2$.

increases with increasing equilibrium protein concentration and in going from the number to the z average. These indicate contamination by nonassociating material (Harris et al., 1969; Teller et al., 1969). Analysis of this data by the two-species plot developed by Horbett (Teller et al., 1969) and Roark (Roark & Yphantis, 1969) and analysis by the Dyson procedure (Van Holde et al., 1969) provided no evidence of protein species other than dimer and tetramer. Thus, dissociation of the Con A dimer to monomeric Con A does not occur. Computer simulation of the data of Figure 1 predicted that a fraction of nonassociating dimers sufficient to cause the microheterogeneity domonstrated in Figure 1A would also decrease the apparent equilibrium constant several orders of magnitude relative to that calculated for Figure 1B. This prediction is not realized. Rather, the pattern of Figure 1B fits nicely with the middle channel of data of Figure 1A, indicating approximately equal apparent equilibrium constants.

Figure 2 demonstrates the effect of the presence of saccharide ligands on the dimer–tetramer equilibrium of Con A which does not contain hydrolyzed subunits. In this case, the molecular weight averages superimpose, independent of the initial protein concentration. The experiments were conducted at 35 °C, a temperature relevant to the application of Con A as an effector of cellular events. The high temperature precluded making measurements at neutral pH, because the dimer–tetramer association constant at pH 7 and 35 °C lies outside the range to which the sedimentation equilibrium technique is sensitive (Senear & Teller, 1981). Consequently, the dimer–tetramer equilibrium was titrated with methyl mannoside at a variety of conditions between pH 5.5 and pH 6.5. The highest methyl mannoside concentration used is sufficient to saturate 99.9% of the Con A–saccharide binding sites according to the binding constant reported by So & Goldstein (1968). It is quite clear in Figure 2 that the self-association of native concanavalin A is not linked to saccharide binding. This result is contrary to an earlier report (McKenzie & Sawyer, 1973). It implies that the presence of saccharide ligands must affect the self-association of Con A dimers that contain hydrolyzed subunits to give the heterogeneity of Figure 1A.

Saccharide binding activity was demonstrated for fragment-free Con A by using methyl mannoside to elute a sample from an affinity matrix of Sephadex G-75 (Olson & Liener, 1967), at pH 6.5. Greater than 90% of the initial sample is

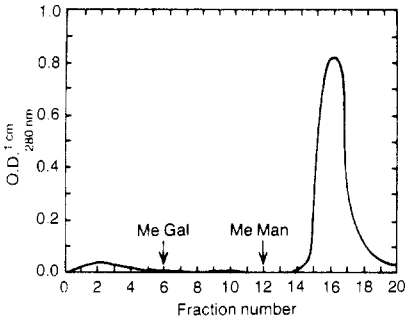

FIGURE 3: Elution of highly purified fragment-free concanavalin A from Sephadex G-75 by 2.0 mM α-methyl mannoside. The column size was 1.5 × 13 cm. A sample of 23.5 mg was loaded onto the column, and 5-mL fractions were eluted with 0.05 M sodium phosphate, pH 6.52, with 0.4 M NaCl and 0.2 mM $CaCl_2$ and $MnCl_2$. The peak accounts for 6% of the starting material. At the arrow marked MeGal, the elution buffer included 2.0 mM α-methyl galactoside, which eluted 1% of the starting material. At the arrow marked MeMan, the elution buffer included 2.0 mM α-methyl mannoside. The peak contains the remainder of the starting material.

specifically eluted by 2.0 mM methyl mannoside (Figure 3), a concentration which is 10-fold less than the maximum concentration in the titration experiments (Figure 2). Less than 1% of the original sample was eluted by methyl galactoside.

Effects of Solvent Composition. The effect of solvent composition on the dimer–tetramer equilibrium of fragment-free Con A was studied to test whether the hydrophobic interactions thought to be the major driving force for the association would show a dependence on the activity of water in solution. For a two-component system consisting of a low molecular weight solute in water, the Gibbs–Duhem relation states that the activity of water is dependent upon the activity of the solute. The effects of $CaCl_2$ and NaCl were studied; $CaCl_2$ because of the dramatic effect of Ca^{2+} on water activity and NaCl because it is not a denaturant, even at extreme concentrations. Also, we expected calcium to favor dimer formation by interacting with carboxylates on the dimer interface and lowered water activity at high $CaCl_2$ concentration to favor tetramer formation, allowing water and $CaCl_2$ effects in the three-component $CaCl_2$–Con A–water system to be resolved.

Since the Con A dimer–tetramer equilibrium involves no secondary reactions such as conformational changes, the free energy of reaction is a function of the chemical potentials (μ) of the components of the solution, $\Delta G = f(\mu_H,\mu_x,\mu_w)$ where H, x, and w refer to protons, nonmacromolecular solutes, and water, respectively. Changes in the reaction free energy are expressed by the total differential

$$d\Delta G = \left(\frac{\partial \Delta G}{\partial \mu_H}\right)_{\mu_x,\mu_w} d\mu_H + \left(\frac{\partial \Delta G}{\partial \mu_x}\right)_{\mu_w,\mu_H} d\mu_x + \left(\frac{\partial \Delta G}{\partial \mu_w}\right)_{\mu_w,\mu_H} d\mu_w \quad (1)$$

Equation 1 can also be written in terms of ln k and ln a_i since $\Delta G = -RT \ln k$ and $\mu_i = \mu_i^0 + RT \ln a_i$. Wyman (1964) demonstrated that $(\partial \ln k/\partial \ln a_i)_{a_{j\neq i}} = \Delta\bar{\nu}_i$, so that the effect on the equilibrium when the activity of only one component is allowed to vary is

$$-\frac{d\Delta G}{d\mu_i} = \frac{d \ln k}{d \ln a_i} = \Delta\bar{\nu}_i = \bar{\nu}_i^{\text{product}} - \bar{\nu}_i^{\text{reactant}} \quad (2)$$

where $\bar{\nu}_i$ is the saturation fraction of component i. The familiar form of the linked function is obtained by substitution of the correct binding expression for $\bar{\nu}_i$ and integration. Equation 2 neglects the terms d ln $a_{j\neq i}$/d ln a_i and so is not exact, but the approximation is sufficiently good to describe; for example, proton binding effects on protein self-association equilibria. Application of the Gibbs–Duhem result to the two-component system composed of a solute, x, and water gives d ln a_w/d ln $a_x = -m_x/m_w$. This shows why the terms d ln $a_{j\neq i}$/d ln a_i can be neglected at low concentrations of component i. Tanford (1969) generalized eq 2 to include the case of high concentrations of x and wrote

$$\frac{d \ln k}{d \ln a_x} = \Delta\bar{\nu}_{pref} = \Delta\bar{\nu}_x - m_x/m_w\Delta\bar{\nu}_w \quad (3)$$

where again, $\Delta\bar{\nu}_i = \bar{\nu}_i^{\text{product}} - \bar{\nu}_i^{\text{reactant}}$. As before, substitution of the proper expressions for $\bar{\nu}_x$ and $\bar{\nu}_w$ and integration should give a useful form of the function. The meaning of the preferential interaction parameter, $\Delta\bar{\nu}_{pref}$, is expressed in the thermodynamically exact three-component notation of Casassa & Eisenberg (1964) by

$$(\partial m_x/\partial m_p)_{\mu_x} = \Delta\bar{\nu}_{pref} \quad (4)$$

where p can refer to either the reactant or product state of the protein. Thus, $\bar{\nu}_i$ is strictly a measure of thermodynamic interaction with no particular mechanism or chemistry implied.[2] However, the nature of the expression substituted for $\bar{\nu}_w$ in eq 3 does make inherent extrathermodynamic assumptions about the nature of water binding. Of the several expressions considered, the simplest makes the assumption that water "binding" sites are always saturated at 55.51 m H_2O, so the $\Delta\bar{\nu}_w$ becomes Δn_w, equal to the difference in hydration between dimeric and tetrameric Con A. With $\bar{\nu}_x = n_x a_x/(K_x + a_x)$ or specific, noninteracting sites for ligand x, this leads to

$$\ln k_2(\text{obsd}) = \ln k_{2,\nu_x=0,\nu_w=0} + n_{T,x} \ln (1 + a_x/K_{T,x}) - 2n_{D,x} \ln (1 + a_x/K_{D,x}) + \Delta n_w \ln a_w \quad (5)$$

where the k_2's are association constants for dimer to tetramer, $K_{T,x}$ and $K_{D,x}$ are dissociation constants for x from the protein in the tetrameric, T, and dimeric, D, states, and $n_{T,x}$ and $n_{D,x}$ are the number of linked sites for ligand x in the tetrameric and dimeric states. Since partial occupancy by water is not considered, this function should give a lower limit to the number of waters removed from the dimer–dimer interface.

Titrations of the Con A dimer–tetramer equilibrium with $CaCl_2$ were performed in a 0.01 M imidazolium chloride buffer, while NaCl and saccharide titrations, as well as previously reported proton titrations (Senear & Teller, 1981), were performed in a 0.05 M sodium phosphate buffer. The data presented in Figure 4, which are typical of the quality obtained in imidazolium chloride buffer under these conditions, confirm that there is no buffer composition effect. These data are best described by a dimer–tetramer model with a dimer molecular weight, M_2 = 51 000. The equilibrium constant calculated, ln k_2(obsd) = 11.37 ± 0.13 root mean square, compared favorably with the prediction from the Wyman analysis of the pH dependence in phosphate buffer (Senear & Teller, 1981), ln k_2(pred) = 11.30.

Association constants obtained as a function of $CaCl_2$ concentration from 0.1 to 2.4 m are presented in Figure 5. It is obvious that calcium favors the dimeric form of Con A within the concentration range studied. The very sharp transition in the pH 6.5 data at ~1.9 m $CaCl_2$ (ln $a_\pm \simeq 0.85$) is of interest. The molecular weight moments observed at these high $CaCl_2$ concentrations were analyzed by the dimer–tet-

[2] For a clear discussion of this point, see Pittz & Timasheff (1978).

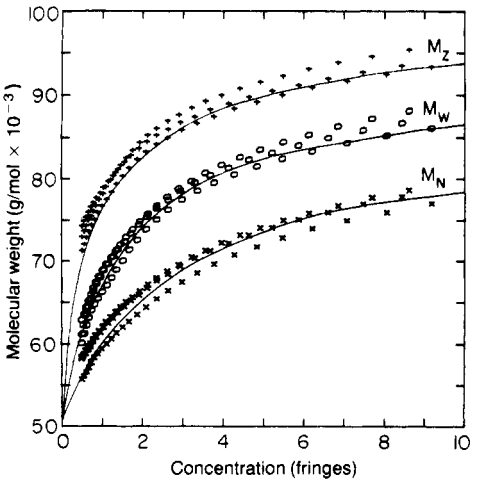

FIGURE 4: Molecular weight distribution of purified fragment-free concanavalin A obtained by sedimentation equilibrium in imidazole–imidazolium chloride buffer. Initial loading concentrations of approximately 0.5, 1.0, and 1.5 mg/mL were centrifuged for 12.5 h at 20 000 rpm and 20 °C in 0.035 M imidazole–imidazolium chloride, pH 6.21, with 0.489 M NaCl and 0.2 mM $CaCl_2$ and $MnCl_2$. 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 = 11.30 ± 0.13 root mean square.

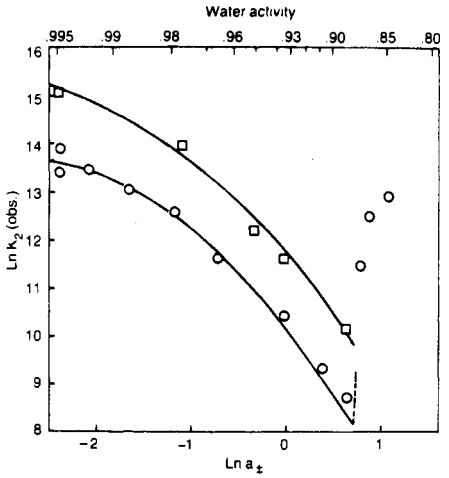

FIGURE 5: Wyman plot of $CaCl_2$ titration data showing natural log of observed equilibrium constant for dimer–tetramer association of concanavalin A as a function of the natural log of mean ionic activity ($a_\pm$) at 25 °C. The points plotted are averages of the estimates of ln k_2 calculated by the two methods described under Experimental Procedures. The symbols are as follows: (□) pH 6.8; (○) pH 6.5. The symbol size indicates the approximate rms deviation. The solid curves are given by the parameters in Table I and represent the least-squares solution of eq 7–9 to these data and to the pH titration data at ln $a_\pm$ = 0.023 (not shown). The dashed line indicates that the dimer–tetramer stoichiometry does not hold above ln $a_\pm \simeq$ 0.8.

ramer model. However, the molecular weight moments were dependent on the initial conditions of the experiment, indicating irreversible behavior. Other analyses provided evidence for the presence of appreciable amounts of polymeric species larger than tetramer. The transition suggests the formation of a second phase. Since secondary reactions such as conformational changes presumably affect the system above the transition point, and since irreversible behavior and at least a partial change in stoichiometry are certainly involved, eq 1 does not apply and the data cannot be analyzed by the linked function theory. Unfortunately, the water activity at this limit is still relatively high, $a_w \simeq 0.87$.

When the data below 1.9 *m* $CaCl_2$, both at pH 6.5 and pH 6.8, were fit by eq 5, no positive value for $K_{T,x}$ was found. The maximum slope, which is a lower bound estimate of the

Table I: Parameters for the Binding of Calcium and Water to Concanavalin A at 25 °C

parameter	eq[a]	value
$pK_{D,x}$	7	0.118
Δn_w	7	0 to −8
$\ln k_{2,\bar{\nu}_w=0,\bar{\nu}_w=0}$	8	17.840
$pK_{D,H}$	9	6.467
rms deviation[b]		0.279

[a] These refer to the equation in the text in which the parameter appears. [b] This refers to the deviation between the observed and predicted ln k_2 values.

number of association-linked calcium sites, indicates n_{Ca} = 4, or one site per subunit. This suggests that the weak binding of calcium to a single site on each subunit of the dimer that becomes buried in the tetramer is linked to the Con A dimer–tetramer equilibrium. The model that fits this scheme is

$$\ln k_2(\text{obsd}) = \ln k_{2,\nu_x=0,\nu_w=0} - 4 \ln (1 + a_\pm/K_{D,x}) + \Delta n_w \ln a_w \quad (6)$$

The Con A self-association is also linked to proton binding (Senear & Teller, 1981), the reaction being governed by the ionization state of a histidyl residue on each subunit. Any ionic strength dependence of the p*K* of this group would constitute a secondary effect of $CaCl_2$ concentration and would systematically affect the data of Figure 5. To ascertain the presence or absence of such an effect, we titrated the dimer–tetramer reaction of Con A with protons at a high concentration of $CaCl_2$ (1.160 *m*). Analysis of the data as described previously (Senear & Teller, 1981) gave $pK_{D,H}^{25\ °C}$ = 6.80 for the linked histidine ionization vs. the previously determined value in phosphate buffer at 0.5 *m* ionic strength, $pK_{D,H}^{25\ °C}$ = 6.37. A Debye–Hückel expression was used to incorporate this effect into the analysis of the $CaCl_2$ titration data. The model used to fit the data in Figure 5 and the pH titration data at 1.160 *m* $CaCl_2$ is

$$\ln k_2(\text{obsd}) = \ln k_{2,\nu_x=0} - 4 \ln (1 + a_\pm/K_{D,x}) + \Delta n_w \ln a_w \quad (7)$$

where

$$\ln k_{2,\nu_x=0} = \ln k_{2,\nu_x=0,\nu_H=0} - 4 \ln (1 + a_\pm/K_{D,H}) \quad (8)$$

and the negative log of the proton dissociation constant is given as a function of ionic strength by

$$pK_{D,H} = pK_{D,H}^\circ + 0.5102 I^{1/2}/(1 + I^{1/2}) \quad (9)$$

A linear term in ionic strength (βI) was considered but was not justified by the improvement in the fit of eq 9 to the data, according to the criterion of Hildebrand (1956). The parameters obtained by this analysis (Table I) confirm the hypothesis that calcium affects the self-association of Con A by binding weakly to a site on each subunit of the dimer and are consistent with the measured p*K* values for the linked histidine group, predicting $pK_{D,H}^{25\ °C}$ = 6.80 at 1.160 *m* $CaCl_2$. Most important, however, is that the minimum in the deviation function occurs at Δn_w = 0 to −8, which is only zero to two linked sites per subunit. Thus, the data below 1.8 *m* $CaCl_2$ is well described by calcium binding and a Debye–Hückel effect on the linked histidine p*K*; it is not necessary to invoke any effect of water–protein interaction.

The issue of solvent composition effects was further examined by repeating the salt titration using NaCl. Since concanavalin A does not salt out in NaCl, data were collected to 6.23 *m*, essentially the solubility limit of NaCl. As before,

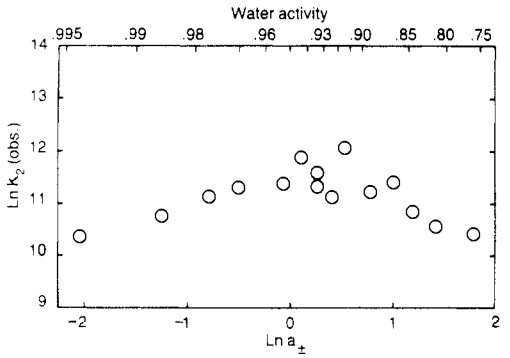

FIGURE 6: Wyman plot of NaCl titration data showing natural log of observed equilibrium constant for dimer–tetramer association of concanavalin A as a function of the natural log of mean ionic activity ($a_\pm$) at 20 °C. The symbol size indicates the approximate rms deviation. Equilibrium constants were calculated as described by Teller (1973) (see Experimental Procedures).

the lower limit to the NaCl concentration was determined by the irreversible aggregation of Con A below ~0.2 M ionic strength. Sedimentation equilibrium data plotted in Figure 6 as ln k_2(obsd) vs. ln $a_\pm$ was well described by the dimer–tetramer model over this range. The quality of the centrifuge data was generally poor. At the very high solution densities reached, the buoyancy term $(1 - \phi'\rho)$ approaches 0 so that small errors in the buffer density calculation create large errors in the apparent molecular weight averages. Very high centrifuge speeds, where rotor vibration becomes a problem, are required. In addition, high centrifuge speeds and high NaCl concentrations cause appreciable redistribution of NaCl. Calculations showed that the effect on the solution density was not sufficient to propagate into a significant error in ln k_2-(obsd). However, the meniscuses in the solvent and solution sectors of the Yphantis cell must be perfectly matched to cancel the effect of this gradient on the Rayleigh patterns produced. All of these contribute to the quality of the data and may have produced systematic errors larger than implied by the root mean square deviations plotted in Figure 6.

The data in Figure 6 indicate that NaCl has essentially no effect on the Con A dimer–tetramer equilibrium. In addition, there is no evidence for any water binding effect, confirming the previous result. At 6.2 *m* NaCl, the water activity is ~0.75, substantially less than the point of the phase transition in $CaCl_2$. The very slight upward trend to the data below ln $a_\pm = 0.5$ is in the direction to be caused by a NaCl effect on the linked histidine p*K*. Analysis of a pH titration performed at 3.31 *m* NaCl gave $pK_{D,H}^{25\ °C} = 5.81$ vs. 6.37 at 25 °C at 0.5 M ionic strength. The fact that the ionic strength dependence of the linked histidine p*K* goes in opposite directions in NaCl and in $CaCl_2$ is attributed to a difference in the slope of the linear term (βI) in the Debye–Hückel formulation. We have observed this with the imidazolium p*K* as well. The downward trend of the data above ln $a_\pm = 0.5$ is in the direction to indicate interaction of NaCl with the protein. The hesitancy to use the term binding is because this interaction, with a p*K* value about −0.5, would account for a standard free energy ($\Delta G° = 300$ cal·mol^{-1}) that is positive.

Discussion

Saccharide Binding Effects. The nature of the heterogeneity demonstrated in Figure 1A is not understood at present. The hypothesis that methyl glucoside binding causes the fraction of Con A dimers that contain hydrolyzed subunits to become incompetent to self-associate was examined by computer simulation of the data in Figure 1A. This led to the conclusion that this hypothesis would explain both the extent of the nonsuperimposability of the molecular weight average distribution curves and the fact that the apparent extent of association is the same in parts A and B of Figure 1, only if the association constant for the dimer–tetramer equilibrium of native dimers were at least 2 orders of magnitude higher in the presence than in the absence of methyl glucoside. It is clear from Figure 2 that no such linkage exists. About 50% of the subunits in the Sigma Con A preparation are hydrolyzed (Senear & Teller, 1981). This is typical of Con A preparations that have not been treated to remove those fragments (Kalb & Lustig, 1968; Cunningham et al., 1972). The fraction is large enought that small differences in saccharide binding activity of native and hydrolyzed subunits can have large effects on the apparent saccharide binding activity of the mixture.

It seems unusual that the self-association of native dimers would not be linked to saccharide binding, even though the saccharide binding sites are over 20 Å from the region of the dimer–dimer interface (Hardman & Ainsworth, 1976). The standard free energy to saturate the tetramer with α-methyl glucoside or α-methyl mannoside is appreciable, on the order of −20 to −24 kcal·mol^{-1}. Taken in the context of the current views on the dynamic nature of proteins, this energy would be expected to make a significant contribution to the number of isoenergetic or nearly isoenergetic conformational states [e.g., Karplus & McCammon (1979)] and to the number of low-frequency vibrational modes (Sturtevant, 1977) available to the dimeric and tetrameric Con A, thereby affecting the free energy difference between them. Relative to its saccharide binding activity, Con A behaves as if it were a rigid rather than a dynamic molecule. Also, the presence of saccharide binding does affect the self-association behavior of dimers containing hydrolyzed subunits. Wang et al. (1975) have shown that the site of the naturally occurring hydrolysis is at serine-118, a residue that is involved in dimer–dimer contacts (Reeke et al., 1975). This suggests some influence of saccharide binding on the region of the dimer interface.

The question of the saccharide binding properties of dimeric and tetrameric Con A has been examined before (McKenzie & Sawyer, 1973). The analysis suffers from the incorrect contention that the association of dimeric to tetrameric Con A is irreversible, but the data, which is not inconsistent with ours, are probably correct and some of the conclusions are instructive. These binding experiments were performed at pH 5, where Con A is largely dimeric, and at pH 7, where it is largely tetrameric (Senear & Teller, 1981). However, these authors could not have separated dimeric and tetrameric Con A as claimed. The fractionation procedure used has been shown to separate the protein into isolated native dimers and a fraction enriched in hydrolyzed subunits (Cunningham et al., 1972). The authors recognized that their supposed dimer fraction was substantially enriched in hydrolyzed subunits. We have shown that dimers containing hydrolyzed subunits are always less competent to self-associate than are native dimers (Senear & Teller, 1981). Thus, McKenzie and Sawyer studied the binding behavior of native Con A and of Con A highly enriched in hydrolyzed subunits. Their conclusion that dimers containing one or two hydrolyzed subunits have less than one saccharide binding site per subunit is probably correct, and it may be true that hydrolyzed subunits do not contain competent saccharide binding sites, a factor to be considered in the interpretation of cell biology experiments involving commercial Con A preparations.

Effect of Solvent Composition. Let us now address the question of the effects of the preferential interactions of Con

A with solution components on the dimer–tetramer equilibrium. It is clear that eq 6 allows a more accurate description of the data in Figure 5 than does the differential form of the Wyman function given by eq 3. Usually, Wyman plots of preferential interaction data are treated by setting the right-hand side of eq 3 equal to the slope of the best straight line through the data points [e.g., Kellet (1971) and Lee & Timasheff (1977)]. The data in Figures 5 and 6 suggest that over a sufficient range of ligand concentration, the relation is, as predicted by eq 6, not a linear one. This nonlinear behavior has also been observed in the case of preferential binding of NaCl, $CaCl_2$, and glycerol to human oxyhemoglobin (N. Ahn and D. C. Teller, unpublished results). The same phenomenon has been observed with other proteins in other solvent systems (Pittz & Timasheff, 1978), and the fact that there can be substantial variations in $\Delta\bar{\nu}_{pref}$ as a function of ligand concentration was shown many years ago (Cohen & Eisenberg, 1968; Reisler & Eisenberg, 1969). These suggest that the nonlinear behavior shown in Figure 5 is general and that an integrated form of the Wyman function is the correct description of such data. Additionally, integration of eq 3 provides a more useful description which expresses preferential interactions as the sum of the various component interactions and avoids the usual ambiguous interpretations of $\Delta\bar{\nu}_{pref}$. For example, in the case of the effect of 1–4 M glycerol on the in vitro reconstitution of tubulin, Lee & Timasheff (1977) observe $\Delta\bar{\nu}_{pref} = 1$ based on the slope of a linear plot. Using eq 3 and referring to 4 M glycerol, they conclude that at least 14 water molecules must be removed from the domain of the protein for each glycerol molecule removed during the reaction. They could as easily have referred to the midpoint of the data, 2.5 M, and suggested a ratio of 22 molecules of water/molecule of glycerol.

A disadvantage of integrating eq 3 is that while eq 3 is perfectly general and simply describes the mutual effects of solution components on the chemical potentials of other components (Casassa & Eisenberg, 1964), extrathermodynamic assumptions are made about the nature of the interaction between components by the form of the function used to describe each $\Delta\nu_i$ term. In the case of specific ligand–protein interactions one defines a thermodynamic equilibrium constant for the reaction $PL_{j-1} + L \rightleftharpoons PL_j$, where P and L refer to protein and ligand, respectively, which leads to the binding isotherm, $\bar{\nu} = n_i k_i L/(1 + k_i L)$. This function describes the effect of calcium on the Con A dimer–tetramer equilibrium. However, it is not clear that this function properly describes the interaction of NaCl and Con A. It is highly unlikely that specific binding of Na^+ to the protein is involved here, since the small NaCl effect observed occurs in the concentration range of 2.5–6.3 *m*, consistent with a "binding" free energy that is insignificant relative to the available thermal energy at 20 °C. Such effects can find a plausible explanation in terms of nonspecific thermodynamic interactions between solution components which need not even come into contact with one another (Lee & Timasheff, 1977).

One's notion of the nature of water binding is similarly restricted by the function used to describe $\Delta\bar{\nu}_w$ in eq 3. The tacit assumption that was made in deriving eq 5 is that any accessible surface constitutes a water "binding site" and that available sites are fully occupied, i.e., full hydration is aqueous solution, independent of a_w. Other functions which might describe the protein–water interaction were also considered. For example, if a_w is identified with the fraction of the total water molecules in solution which are not hydrating low molecular weight solutes, e.g., Ca^{2+}, and if n_w and ν_w are identified with the protein hydration in pure water ($a_w = 1$) and the protein hydration at any $a_w < 1$, respectively, then the expression can be derived

$$\nu_w = \frac{n_w a_w C}{1 + (C - 1)a_w} \tag{10}$$

where $C = 1 + k/55.51$ and the standard free energy for the reaction $PW_{n-1} + W \rightleftharpoons PW_n$ is given by $\Delta G° = -RT \ln k$. This is similar to the function derived by Brunauer et al. (1938) to describe the absorption of gases in multimolecular layers to solid surfaces. It defines that all water binding sites be exactly fully saturated in and only in the pure solvent, making $\Delta\bar{\nu}_w$ a function of protein concentration as well as a function of the concentration of any third component. D. C. Teller (unpublished observations) has successfully described the effect of hydration on the activity coefficients of sugars and some other nonelectrolytes in aqueous solution in this manner. When this function was used to analyze the data in Figure 5, the least-squares fit to the points was excellent with $\Delta\bar{\nu}_w = 0$ to −36, which is from zero to nine waters released per subunit. However, in no case did the program find a value for $C \geq 1$. C must be ≥ 1 so that k is positive; values < 1 are not thermodynamically meaningful. Thus, the validity of the conclusion that water binding effects need not be invoked to explain the $CaCl_2$ and NaCl data remains.

The fact that the water molecules that must be released from the dimer–dimer interface region do not affect the apparent thermodynamics of the reaction is placed more clearly in context when the number of water molecules involved is considered. We have made three independent estimates of this number. First, the effect of preferential interactions with solution components on the apparent specific volume (ϕ') of a protein in solution is expressed in the three-component notation of Casassa & Eisenberg (1964) by

$$1 - \phi'\rho = 1 - \bar{\nu}\rho + x_{pref}(1 - \bar{\nu}_x\rho) \tag{11}$$

where $\bar{\nu}$ and $\bar{\nu}_x$ are the protein and low molecular weight solute partial specific volumes, ρ is the solution density, and x_{pref}, which is the preferential interaction parameter expressed in gram units, is

$$x_{pref} = M_x/M_p(\nu_x - m_x\nu_w/m_w) \tag{12}$$

The values of the apparent specific volume of Con A that were observed as a function of $CaCl_2$ concentration were analyzed by eq 11 and 12 where the calcium binding parameters that were determined from the data in Figure 5 were substituted for ν_x. The number of waters calculated, ν_w, was apportioned between the dimer–dimer interface and the rest of the surface of the Con A tetramer on the basis of the fractional change in accessible surface area [calculated in a manner similar to that of Shrake & Rupley (1973) by using the coordinates of Reeke et al. (1975)] during the dimer to tetramer transition. An estimate of Δn_w equal to 150–300 was obtained. Second, the number of water molecule sized beads necessary to coat the accessible surface that is buried in going from dimer to tetramer to a density corresponding to roughly 1 g·mL^{-1} was calculated. This gave an estimate of Δn_w equal to 150–200. Third, previously we reported a heat capacity change for the reaction, $\Delta C_p = -821$ cal·mol^{-1}·deg^{-1}, of which −720 cal·mol^{-1}·deg^{-1} might be due to the release of water from the surface of the protein (Senear & Teller, 1981). From Suurkuusk's (Suurkuusk, 1974) specific heat data, it can be concluded that there is an average heat capacity change of −5.4 to −5.6 cal·deg^{-1}·(mol of water released)$^{-1}$. Yang & Rupley (1979) estimate −5.8 cal·mol^{-1}·deg^{-1} from their lysozyme heat

capacity data. Thus, the heat capacity data are consistent with the release of 125–130 water molecules during the dimer–tetramer transition.

According to Tanford's extension of the Wyman theory, what is in effect a 125–300th order condensation of water on the freshly exposed surface of the Con A dimer during the tetramer to dimer transition should have a profound effect on the apparent equilibrium constant over the range of water activity, $1 \geq a_w \geq 0.75$. The fact that no such effect is observed with Con A in $CaCl_2$ or in NaCl and with human oxyhemoglobin in $CaCl_2$, NaCl, and glycerol (Ahn and D. C. Teller, unpublished experiments) calls into question this notion of preferential interactions. It suggests that while one can formally write $\Delta\nu_{pref} = \Delta\nu_x - m_x/m_w\Delta\nu_w$, $\Delta\nu_{pref} = \Delta\nu_x$ is what is observed. This does not mean to suggest that proteins are not preferentially hydrated under high-salt conditions. After all, Cox & Schumaker (1961a,b) showed 20 years ago that preferential hydration can be measured as an apparent specific volume effect. It only suggests that Tanford's linkage equation does not properly express the effect of preferential hydration on protein self-association equilibria.

Finally, the linkage of the Con A dimer–tetramer equilibrium with weak calcium binding is of interest. That NaCl has no effect implicates calcium rather than chloride and suggests a specific ion site rather than simply an arrangement of negative charges which might provide a general cationic site. This weak calcium site should not be confused with the site that is observed in electron-density maps (Hardman & Ainsworth, 1972; Reeke et al., 1975). That site, which is involved in saccharide binding, has a dissociation constant on the order of 10^{-3} M (Kalb & Levitski, 1968) and should be well saturated at the lowest $CaCl_2$ concentration used here. Calcium does not bind to the linked site in the tetramer, suggesting a site that is on the dimer–dimer interface. The weakness of the interaction ($\Delta G° \simeq -0.1$ kcal·mol^{-1} at 25 °C) makes it unlikely that long-range structural effects are involved.

Using the coordinates of Reeke et al. (1975), we have calculated the accessibility of all surface residues of the Con A protomer, of both the I–II and I–III dimers [numbering scheme of Reeke et al. (1975)] and of the tetramer. Two carboxylate side chains, those of glutamate-192 and aspartate-58, are accessible in Reeke's solution dimer (the I–II dimer) but are completely buried in making the I–III interaction (the tetramer). No carboxylate side chain that is accessible to a probe the size of a hydrated calcium ion in the protomer is buried in making the I–II pair. Thus, the linkage of calcium binding and the self-association is consistent with the identification of the I–II dimer as the solution dimer. While it seems likely that either glutamate-192 or aspartate-58 is the linked calcium binding site, it cannot be both, first, because the groups are 15 Å apart and, second, because the calcium binding is several orders of magnitude too weak. We have previously shown that the dimer–tetramer equilibrium is linked to proton binding by a histidyl residue in the dimer–dimer interface, probably histidine-51. Histidine-51 (of subunit I) is part of a cluster of charged groups that form interdimer electrostatic interactions and which includes also lysine-114 and -116 of subunit II and glutamate-192 of subunit I (Reeke et al., 1975). We hypothesized that proton binding to histidine-51 would result in a high density of positive charge balanced by a lone negative charge. It is an intriguing possibility that Ca^{2+} binding by glutamate-192 would produce the same effect, although there is no compelling evidence on the matter.

In conclusion, we have demonstrated that, contrary to the conclusion reached by McKenzie & Sawyer (1973), saccharide binding does not affect the dimer–tetramer equilibrium of native concanavalin A. It does introduce irreversible behavior to preparations containing significant proportions of hydrolyzed subunits. The dimer–tetramer equilibrium is linked to the weak binding of calcium to a site probably in the dimer–dimer interface. There is no evidence for specific effects of hydration on the dimer–tetramer equilibrium as are implied by Tanford's extension of the Wyman linked function theory.

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Calorimetric Study of the Interaction of Lysozyme with Aqueous 1-Propanol†

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ABSTRACT: The enthalpies of transfer of hen egg white lysozyme from water to aqueous solutions of 1-propanol were determined by isothermal flow calorimetry at 10, 17, 25, and 40 °C in 0.04 M, pH 2 glycine buffer. Alcohol concentrations up to 3.4 M were employed. Four regions in the dependence of the enthalpy of transfer on alcohol concentration can be discerned: a region of linear increase observable at 10, 17, and 25 °C, an inflection region observable at 17 and 25 °C, a second linear region observable at 17, 25, and 40 °C, and a region of decreasing enthalpies seen at 40 °C. Combination of differential scanning calorimetric data on lysozyme in PrOH–H_2O mixtures [Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180–1186] with the transfer enthalpies reported here shows that the enthalpy in this system can be regarded as a state function and that the apparent specific heat is at first slightly decreased and then strongly increased by the addition of 1-propanol. Comparison of the results for the interaction of lysozyme with 1-propanol with those reported for the interaction with guanidine hydrochloride [Pfeil, W., & Privalov, P. L. (1976) *Biophys. Chem. 4*, 23–50] indicates that the denaturing effects of these two reagents involve very different mechanisms.

We have previously reported (Velicelebi & Sturtevant, 1979) the results of an extensive DSC[1] study of the thermal denaturation of lysozyme in aqueous mixtures of MeOH, EtOH, and PrOH. It was shown that the unfolding of the protein in these solvent systems is reversible and that the calorimetric and van't Hoff enthalpies of denaturation are nearly equal, indicating that the transition under equilibrium conditions is essentially two state in character. In this paper we present the results of isothermal flow calorimetric determinations of the enthalpies of interaction of lysozyme with PrOH at 10, 17, 25, and 40 °C. PrOH was selected as the alcohol to be used in this study since it is much more effective than either MeOH or EtOH in altering the denaturational behavior of the protein.

Materials and Methods

Hen egg white lysozyme (*N*-acetylmuramide glycanohydrolase; EC 3.2.1.17) was obtained from Sigma Chemical Co. as grade 1, 3 times crystallized, dialyzed, and lyophilized powder. This product was further treated by dissolution in and dialysis against 0.05 M ammonium formate buffer at pH 4.0 and subsequent lyophilization. All buffer solutions were prepared from analytical grade reagents. Protein solutions were prepared by dissolving the lyophilized protein in degassed buffer, the concentrations being determined spectrophotometrically by using $E_{280}^{1\%} = 26.5$ in aqueous solution (Bjurulf & Wadsö, 1972). The absorbance measurements were carried out at 10 °C where the protein was native in all the alcohol solutions employed as judged by DSC denaturation profiles. All the calorimetric experiments were carried out in 0.04 M glycine buffer adjusted in the presence of PrOH to pH 2.0 as

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received August 12, 1980.* This research was aided by grants from the National Institutes of Health (GM-04725) and the National Science Foundation (PCM76-81012).

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[1] Abbreviations used: DSC, differential scanning calorimetry; MeOH, methanol; EtOH, ethanol; PrOH, 1-propanol; Gdn·HCl, guanidine hydrochloride.

0006-2960/81/0420-3091$01.25/0