detected by crosslinking the species with glutaraldehyde. For many of the experiments ^{125}I-labeled *C* subunits were used; thus,"stopping"the assembly by the addition of excess inactive C_S chains (produced by dissociation of C_S trimers) permitted us to determine how much protein had already been incorporated into C_N trimers before the "chase." The monomers present at the time of the addition of c_S were assembled into hybrids which migrated to different positions upon electrophoresis in polyacrylamide gels. Dimers were determined by electrophoresis experiments in gels containing sodium dodecyl sulfate; for these experiments the reconstitution was"stopped"by the addition of glutaraldehyde which crosslinked trimers efficiently and rapidly.

The half-time for reactivation of the urea-denatured species was 50 min at 0°C and was independent of concentration from 0.1 to 0.4 mg/ml. First-order kinetics was observed in all experiments. Moreover, electrophoretic determinations of the formation of C_N trimers in the experiments stopped with c_S showed that the rate of assembly of trimers was identical to that for the restoration of enzyme activity.

Reactivation of the NaSCN-dissociated protein was much more rapid than that for the urea-denatured protein. At a concentration of 0.37 mg/ml the half-time was 5 min and it increased as the protein concentration was lowered. Data from experiments over a limited concentration range fit second-order kinetics and the crosslinking experiments showed that the predominant species throughout the assembly process were trimers and monomers; the dimer concentration was less than 5% (of the total protein).

These results allow us to describe the events in the assembly of *C* subunits. Since the rate of reactivation of the urea-denatured species is identical to the rate of trimer formation, the rate-limiting step must be a folding of the individual chains which must occur before association can take place. In contrast, the half-time for reactivation of the NaSCN-denatured protein is concentration-dependent; hence the relatively folded monomers present in the solution immediately after dilution of the perturbant have very little enzyme activity. During the course of the assembly process, dimers do not accumulate to a significant concentration, indicating that folded monomers associate to form dimers which rapidly associate with other monomers to form trimers.

In summary, it appears that the first step in the assembly of the *C* subunits is the folding of unstructured chains to form inactive (or at most, only partially active) monomers. These monomers then associate to form dimers which rapidly combine with other monomers to form stable, active trimers.

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THERMODYNAMIC STUDIES OF CONCANAVALIN A DIMER-TETRAMER EQUILIBRIA

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We have studied the reversible dimer-tetramer association equilibria of the jack bean lectin concanavalin A. Equilibrium constants were measured by the high speed sedimentation

TABLE I

THERMODYNAMIC PARAMETERS OF CONCANAVALIN A ASSOCIATION AT 25° AND 0.5 M IONIC STRENGTH, AND COMPARISON OF THE IONIZATION PARAMETERS WITH IMIDAZOLE

Constant*	$\log_{10} k_i$	ΔH°(kcal/mol)	ΔS°(e.u.)	ΔCp(cal/mol·degree)
k_o‡	6.87	10.4	66.3	-820
k_H	6.47	-7.6	+3.7	0
Imidazole§	6.99	-8.8	+2.6	-0.45

Note that the ionization thermodynamics are written for the association reaction, $B + H^+ \rightarrow BH^+$.

*Constant refers to the parameters of Eq. 2.

‡Huet (3) found $\log_{10} k = 7.38$, $\Delta H° = 39$ kcal/mol, $\Delta S° = 160$ e.u. at 23°

§From reference 4.

equilibrium technique as a function of pH, temperature, and $CaCl_2$ concentration. Both commercial and purified[1] preparations of concanavalin A were studied.

Equilibrium constants for the pH and temperature dependence of the reaction were analyzed as Wyman linked functions (2) using a truncated van't Hoff temperature dependence. From 4° to 37°C and between pH 5.5 and 7.5 only dimer and tetramer species were present. The model that fit these data is given by the chemical equilibria:

$$\begin{array}{ccc} & 2D \rightleftharpoons T & \\ 4H^+ & \rightleftharpoons & 4H^+ \\ & 2DH_2^+ & \end{array}$$

where T represents tetramers and DH^+_2 and D represent protonated and unprotonated dimers. This scheme is described by Eq. 1:

$$ln\ k_{obs} = ln\ k_o - 4\ ln(1 + [H^+] \cdot k_H), \quad (1)$$

where $ln\ k_i = a_i + b_i/T + c_i\ ln\ T$ and k_{obs}, k_o, and k_H refer to the observed association constant, the association constant at zero proton binding, and the association constant for protons, respectively. The six coefficients were fit to the observed equilibrium constants by nonlinear least squares techniques, and used to calculate the thermodynamic parameters in Table I.

It is clear from Table I that the ionization thermodynamics are consistent with the ionization thermodynamics for the imidazolium group. The small heat capacity and small entropy are inconsistent with a carboxylate ionization while the enthalpy is too small for an amine ionization. Given the identification of the protomers composing the dimer by Reeke (5), histidine 51 or histidine 121 is the residue most likely responsible for the ionization thermodynamics. Both are accessible in the dimer, both are completely buried in the tetramer, and both are near positively charged groups in the opposite dimer.

The association of purified concanavalin A proceeds with the large entropy and heat capacity changes typical of hydrophobic reactions (Table I) but with a large positive enthalpy as well. This is consistent with crystallographic studies which show that the region of

[1]About 50% of concanavalin A subunits in commercial preparations are hydrolyzed between residues 118 and 119. These can be precipitated by ammonium bicarbonate at 37°C to yield a preparation consisting only of intact concanavalin A subunits (1).

dimer-dimer contact is the large, hydrophobic, antiparallel β-pleated sheet region that forms the back of the dimer. In the dimer, this structure is completely exposed to solvent.

Contrary to an earlier report (6), the association of purified concanavalin A is not linked to α-methylmannoside or α-methylglucoside, two sugars which bind to concanavalin A. However, the presence of either sugar increases the difference between the association of dimers composed of intact chains and dimers composed of fragmented chains. That sugar binding should not be linked to self-association for intact concanavalin A is surprising for two reasons. First, the free energy of binding for the mannoside and glucoside is appreciable, of the order -5 to -6 kcal/mol. It seems unusual that the strain involved in distributing this energy should not be manifested in small changes in the dimer-dimer interface. Second, the decrease in the degree of association for the fragmented chain fraction in the presence of sugar argues for a linkage.

Tanford (7) has extended the Wyman linked function to include the preferential interactions of solution components with a macromolecule. Integration of Tanford's equation allows preferential interactions to be described as the sum of salt (x) and water (w) binding, but requires extrathermodynamic assumptions about the nature of water binding. We have assumed that water is always saturating in aqueous solution so that water binding becomes a constant equal to the number of water sites (n_w) created or destroyed in the association. This gave Eq. 2:

$$ln\ k_{obs} = ln\ k^{\circ} - n_x\ ln(1 + a_x \cdot k_x) - n_w\ ln\ a_w, \quad (2)$$

where a_i represents activity and the k's are association constants as in Eq. 1. L$n\ k^{\circ}$, n_x, k_x, and n_w were fit to the $ln\ k$ vs. $ln\ a_x$ data by nonlinear least squares techniques.

The effect of calcium (from 0.2 to 2.2 M) was studied because the activity of water is low in these solutions. A Debye-Hückel term was added to Eq. 2 because a pH titration at 1.0 M $CaCl_2$ demonstrated a pK shift of the residue responsible for the pH dependence of the reaction. The association of concanavalin A in $CaCl_2$ is accompanied by the loss of four calcium ions and 0 to 8 water molecules per tetramer. Acquisition of calcium sites in the dimer supports Reeke's identification (5) of the protomers composing the dimer.

The number of waters observed is small relative to the number needed to hydrate the large dimer-dimer contact area or even relative to the number of hydrogen bonded waters expected in this region. A sharp transition at 1.4 M $CaCl_2$, above which concanavalin A begins to salt out of solution, limits the range of the data. At this $CaCl_2$ concentration, the water activity may still be too high ($\simeq$0.91) to observe the effect of water binding.

Under the conditions of cell culture experiments, purified concanavalin A at 100 μg/ml is 95% in the tetrameric state. However, dimers composed of intact polypeptide chains associate more strongly than do those with fragmented polypeptide chains.[1] Appreciable amounts of dimer are probably present in commercial concanavalin A preparations under these conditions.

In summary, we conclude that histidine 51 or 121 is the ionizable group that governs the self-association of concanavalin A, and that calcium ions bind to the dimer-dimer interface.

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DOUBLE FILAMENTS IN FIBERS AND CRYSTALS OF DEOXYGENATED HEMOGLOBIN S

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INTRODUCTION

Sickle cell hemoglobin (HbS) molecules in solution or in SS erythrocytes (those from individuals homozygous for the sickle hemoglobin gene), when deoxygenated, aggregate to form fibers that pack into paracrystalline arrays. The diminished oxygen affinity of HbS is produced by the polymerization, and the distortion of the pliant erythrocyte membrane in sickle cell disease results from the elongation of polymers and their subsequent alignment. One of the important problems to be solved in sickle cell disease is the definition of the intermolecular interactions that stabilize the fiber structure. Knowledge of these interactions might lead to the design of stereospecific antisickling agents for clinical use that could inhibit polymerization or could at least destabilize the fiber.

RESULTS AND DISCUSSION

In general, molecular structure and fiber architecture must first be known to find the interactions of one molecule with its neighbors in fiber or in other helical structures. In this particular case, since the structure of deoxy-hemoglobin S is known to 3 Å resolution (1), and since the fiber diffraction pattern shows remarkable similarity to the patterns of monoclinic crystals of deoxy-HbS (form I) (2), the interactions can be determined without complete knowledge of molecular packing in the fiber. By comparing fiber and crystal diffraction patterns, we have been able to establish that the basic structural unit of the fiber consists of double filaments. As in the crystal, one filament is related to its neighbor by the operation of a twofold screw axis located between the filaments and parallel to the fiber axis. Establishment of the partial structure of the fiber leads to the identification of interactions along and between the filaments similar to those in the crystal. The major stabilizing interaction is between the substituted amino acid residue in HbS, Val$1\beta 6$, on one molecule and the hydrophobic residues Phe$\beta 85$ and Leu$\beta 88$ on the neighboring molecule in the adjacent filament (3). Within each filament, hydrogen bonds and van der Waals forces account for most of the interactions.

Another monoclinic crystal (form II) has been identified in capillaries of tightly packed