Hydrodynamic Changes Accompanying the Loss of Metal Ions from Concanavalin A

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The hydrodynamic changes which accompany the dissociation of metal ions from concanavalin A at acid pH are a result of charge effects rather than of dissociation of metal ions as such. Measurements of the rotational relaxation time are discussed in terms of the hydration of the protein and its polymeric heterogeneity.

Concanavalin A, the phytohaemagglutinin from jack bean (*Canavalia ensiformis*), has a variety of biological actions and has become an important tool for immunologists and cell biologists (Chowdhury & Weiss, 1975). Each monomer unit (mol.wt. 27000) binds one molecule of carbohydrate and two metal ions [Ca(II) and Mn(II)]. The metal-ion-binding sites are close together [0.45 nm (4.5 Å)], but are topographically distinct from the carbohydrate-binding site (Edelman *et al.*, 1972; Hardman & Ainsworth, 1972).

The binding of carbohydrate induces minor conformational perturbations which can be monitored by circular dichroism and absorption-spectroscopic methods (Pflumm *et al.*, 1971; Hassing & Goldstein, 1970). These perturbations are not large enough, however, to change the hydrodynamic properties of the protein (McKenzie *et al.*, 1972; Inbar *et al.*, 1973). A conformational transition has also been detected on dissociating the metal ions from the protein at acid pH (McKenzie *et al.*, 1972; Sawyer *et al.*, 1974). In the present communication we describe hydrodynamic changes which accompany this dissociation and determine whether they result from the dissociation of metal ions as such or from a more general effect of pH and molecular charge.

Materials and methods

Concanavalin A was prepared from jack-bean meal (lot 100c-5020; Sigma Chemical Co., St. Louis, Mo., U.S.A.) by affinity chromatography (Agrawal & Goldstein, 1967). 5-Dimethylaminonaphthalene-1-sulphonyl chloride (Dns chloride) was purchased from Sigma. Dansylation of concanavalin A was carried out in phosphate (0.02 M-NaH₂PO₄, 0.027 M-Na₂HPO₄) buffer at pH 7.0 rather than at higher pH values, where the protein is irreversibly denatured. The

weight ratio of Dns chloride to concanavalin A was 1:42 and the reaction was allowed to proceed at 3°C for 24 h. The material was passed through a column (1 cm × 20 cm) of Dowex 2 (X8) to remove the majority of free Dns chloride and was then dialysed extensively for 3-4 days against frequent changes of the appropriate buffer to remove minor traces of Dns chloride. Complete removal of free Dns chloride could not be accomplished at pH 5.0 but only at pH 7.0 or 2.1. The degree of labelling was determined spectrophotometrically at 340 nm by using an extinction coefficient of $3.37 \times 10^7 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Chen, 1968). The concentration of the dansylated protein was determined refractometrically by using a value of 0.188 ml/g as the specific refractive increment. Preparations contained between 0.6 and 0.9 mol of Dns/mol of concanavalin A monomer. The conjugate retained its ability to bind carbohydrate as determined by its retention on Sephadex G-50. The apoprotein was prepared by dialysis at pH 2.1 in the presence of 1 mM-EDTA; it was examined at this pH or adjusted to pH 5.0 by dialysis against acetate (0.1 M-sodium acetate, 0.029 M-acetic acid) buffer.

Sedimentation-velocity experiments were performed with a Spinco model E analytical ultracentrifuge. The percentage of dimer and tetramer in samples of concanavalin A was determined by graphical resolution of schlieren profiles as described previously (McKenzie *et al.*, 1972). In experiments where the sedimentation of the protein was examined at the same concentration, but in two different solvents (Fig. 1), two cells were run in the same rotor to ensure comparison at the same speed and temperature.

The hydration of concanavalin A was measured at pH 2.1 and 5.0 by a low-temperature proton-magnetic-resonance technique (Kuntz, 1971a,b). The method

measures the proton signal of unbound water at temperatures below freezing. A Jeol model JNM-4H-100 100MHz instrument was used and spectra were recorded at -30°C .

Fluorescence polarization measurements were recorded with a Hitachi-Perkin-Elmer MPF-3 spectrofluorimeter. Fluorescent lifetimes (τ) were measured with a nanosecond decay instrument described previously (Selinger & Knight, 1973). Rotational relaxation times (ρ) were calculated from the Perrin (1934) equation [eqn. (1)] by using values of τ obtained from the nanosecond decay measurements.

Values of ρ were related to a theoretical value according to the following considerations. For a spherical molecule, the polarization (p) is given by:

$$\left(\frac{1}{p} \pm \frac{1}{3}\right) = \left(\frac{1}{p_0} \pm \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right) \quad (1)$$

where p_0 is the polarization applicable in the absence of rotational motion. The theoretical relaxation time for a sphere of given molecular weight (M), partial specific volume ($\bar{v}_2$) and hydration (δ) is given by:

$$\rho_0 = \frac{3\eta M}{RT} (\bar{v}_2 + \delta \bar{v}_0) \quad (2)$$

where η is the viscosity of the solvent and $\bar{v}_0$ the partial specific volume of water. For a non-interacting mixture of dimeric and tetrameric macromolecules, we may calculate a theoretical weight-average relaxation time ($\bar{\rho}_0$) if it is assumed that the partial specific volumes of the two species are similar and that, as a first approximation, both species have spherical symmetry.

$$\bar{\rho}_0 = \frac{C_D \rho_D + C_T \rho_T}{C_D + C_T} = \frac{\rho_D (C_D + 2C_T)}{C_D + C_T} \quad (3)$$

where C_D and C_T are the weight concentrations of dimer and tetramer respectively.

Results and discussion

The dependence of the sedimentation coefficient on the concentration of concanavalin A at pH 5.0 and 1.7 is shown in Fig. 1. The experiments were carried out at high ionic strength (0.3M) to minimize the primary charge effect (Pedersen, 1958). The dependence is linear, and least-squares fit of the data provided values for $s_{20,w}^0$ of 3.99S at pH 5.0 and 3.77S at pH 1.7. The frictional coefficient calculated from the Svedberg equation increased from 5.97×10^{-8} at pH 5.0 to 6.28×10^{-8} at pH 1.7. The molecular weight remains constant (McKenzie *et al.*, 1972).

The hydration of concanavalin A at pH 5.0 and 2.1 was 0.37 ± 0.01 and 0.32 ± 0.02 g/g respectively. The value at pH 5.0 is within the range of theoretical

Fig. 1. Dependence of the sedimentation coefficient on the concentration of concanavalin A

○, pH 5.0; △, pH 1.7. Buffer compositions were as follows: pH 5.0, 0.1M-sodium acetate, 0.029M-acetic acid, 0.2M-NaCl; pH 1.7, 0.023M-HCl, 0.268M-NaCl.

values calculated on the basis of amino acid composition and the hydration of individual residues (Kuntz, 1971a,b). The theoretical value is between 0.35 and 0.39 g/g depending on the ratios assumed for aspartic acid:asparagine and glutamic acid:glutamine. The decrease in hydration at pH 2.1 is attributed to the lower hydration of un-ionized carboxyl groups.

The results of fluorescence experiments are summarized in Table 1. Although concanavalin A is dimeric at pH 5.0, it exists as a non-interacting mixture of dimer and tetramer at pH 7.0 (McKenzie *et al.*, 1972). As the dansylation was carried out at pH 7.0, it was important to determine the degree of reversibility of the association on adjusting solutions to lower pH values. The ratio of dimer to tetramer was determined at each pH value by sedimentation-velocity analysis, and the results are shown in Table 1, column 2. The decay of fluorescence was heterogeneous, as might be expected for heterogeneous labelling and the presence of more than one species. The lifetimes shown in Table 1, column 3, are those representing 70% of the decay intensity. The rotational relaxation-time ratio ($\rho/\bar{\rho}_0$; Table 1, columns 6 and 8) compares the experimental relaxation time with that expected for molecules of the same molecular weight and hydration as concanavalin A, and takes into account the proportion of dimer and tetramer forms present according to eqn. (3).

With respect to these results we make the following observations.

1. The increase in the relaxation-time ratio and the frictional coefficient at acid pH values indicates an increase in asymmetry or an expansion of the molecule. The phenomenon appears to result from a

Table 1. *Experimental and theoretical rotational relaxation times (ns) for concanavalin A-Dns conjugates*

Buffer compositions were as follows: pH2.0, 0.0095M-HCl, 0.0905M-NaCl, $I = 0.1M$; pH5.0, as for Fig. 1; pH7.0, 0.02M- NaH_2PO_4 , 0.027M- Na_2HPO_4 , $I = 0.1M$.

pH	Weight ratio dimer:tetramer	τ^*	$\rho^\dagger$	Unhydrated		Hydrated	
				$\bar{\rho}_0^\ddagger$	$\rho/\bar{\rho}_0^\ddagger$	$\bar{\rho}_0^\ddagger$	$\rho/\bar{\rho}_0^\ddagger$
2.1	100:0	20	139	44.7	3.11	62.7	2.22
2.1 $\rightarrow$ 5.0§	100:0	13	52	44.7	1.17	62.7	0.83
2.1 $\rightarrow$ 5.0, plus Ca^{2+} and Mn^{2+}	100:0	13	54	44.7	1.21	62.7	0.86
7.0	60:40	17	81	63.0	1.29	87.8	0.92

* Lifetime characteristic of 70% of the intensity.

† Rotational relaxation time at 25°C.

‡ Theoretical relaxation times calculated according to eqns. (2) and (3).

§ Apoprotein at pH5.0.

|| Apoprotein at pH5.0, plus 1mM- $CaCl_2$ and 1mM- $MnCl_2$.

charge effect rather than from the dissociation of metal ions as such, since the relaxation-time ratio of the apoprotein at pH5.0 is similar to that of the native protein. Restoration of the metal-ion content, by the addition of calcium and manganese salts to the apoprotein at pH5.0, did not change the relaxation-time ratio.

2. Approximately 35% of the relaxation time can be attributed to the volume occupied by the water of hydration, and failure to take it into account can lead to an overestimate of the relaxation-time ratio. However, there remains the uncertainty as to the degree to which water of hydration affects rotational characteristics of the macromolecule (Kuntz & Kauzmann, 1974).

3. The value of the relaxation-time ratio at pH5.0 and 7.0 ($\rho/\bar{\rho}_0 \approx 0.9$) suggests that there is little internal flexibility or asymmetry in the molecule. It is possible, however, that these effects exactly counter-balance each other to give a relaxation-time ratio close to unity. We believe that such a situation is unlikely in concanavalin A, since X-ray studies have shown that the molecule is compact and approximately spherical, and the high proportion of β -structure (approx. 55%) would preclude flexibility of polypeptide segments or domains. Moreover, the articulation of monomer units within a dimer is unlikely, since the β -sheets are continuous across the monomer-monomer plane of interaction (Edelman *et al.*, 1972; Hardman & Ainsworth, 1972).

4. The volume of the unhydrated tetramer of concanavalin A obtained from measurements of the 0.2nm (2Å) electron density map is $1.35 \times 10^2 \text{ nm}^3$ ($1.35 \times 10^5 \text{ Å}^3$) (Edelman *et al.*, 1972). Hydration of 0.37g/g would increase this volume to $1.93 \times 10^2 \text{ nm}^3$. The relaxation time for a 60:40 mixture of dimer and tetramer at pH7.0 was 81ns (Table 1): used in conjunction with eqn. (3), the relaxation time of the tetramer is 125ns. This in turn provides a value for the hydrated tetramer of $1.91 \times 10^2 \text{ nm}^3$, in good agreement with that above. A slightly

higher value ($2.5 \times 10^2 \text{ nm}^3$) was obtained by Yang *et al.* (1974) from direct measurement of the anisotropy of decay. Inbar *et al.* (1973) obtained a value of 58ns for the relaxation time of concanavalin A at pH7.2. However, the proportion of dimer and tetramer in the preparation was not determined, and the value cannot be related to a theoretical rotational relaxation time.

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