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# Physico-chemical and thermodynamic properties of monomeric Concanavalin A

**E. Battistel[1], G. Lazzarini[1], F. Manca[2], and G. Rialdi[1]***

[1] Centro Studi Chimico Fisici Macromolecole Sintetiche e Naturali, C.N.R.-Corso Europa 30, I-16132 Genova, Italy
[2] Cattedra di Immunologia. Universita' di Genova, Viale Benedetto XV, I-16132 Genova, Italy

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**Abstract.** An alkylated monomer of Concanavalin A was prepared photochemically according to the method of Tanaka et al. (1981). Its affinities for methyl-α-D-gluco, methyl-α-D-manno and p-nitrophenyl-α-D-manno pyranoside were calculated. The enthalpies of these binding reactions were measured calorimetrically and the thermodynamic parameters were calculated. The values obtained suggest that the structure of the monomer differs from that of the dimeric and tetrameric molecules.

Calorimetric studies also showed that the monomeric derivative reacts with IgM but not IgG. The enthalpy per binding site in the monomer-IgM reaction is equal to that of the monomer-mannose derivative reaction; mannose is the terminal residue of the saccharide chains of the IgM molecule. The stoichiometry of the reaction is ten ConA-m per IgM molecule.

**Key words:** Calorimetry, Concanavalin A, photoalkylation, immunoglobulins

## Introduction

Concanavalin A[1] (ConA), a protein isolated from jack bean is an extensively studied lectin which binds α-D-gluco and α-D-mannopyranosides over a pH range from 4 to 8. The thermodynamic properties of its binding to sugars (Munske et al. 1978, 1984; Dani et al. 1981) and immunoproteins (Dani et al. 1982) have already been studied; each subunit has one independent saccharide binding site. At room temperature and pH 7.5 the protein is a tetramer but at pH lower than 5 it undergoes a reversible change to the dimer form which has a molecular mass of 54,000. Sedimentation equilibrium methods have been used to analyze the dimer-tetramer association as a function of pH (Senear and Teller 1981) and at different salt and urea concentrations (Herskovits et al. 1983).

A monovalent derivative of a monomer ConA molecule[2] (ConA-m) has been obtained by photochemical alkylation (Tanaka et al. 1981). In this paper we report the thermodynamic parameters of the reaction of sugars and immunoproteins with this alkylated derivative.

We found that ConA-m has the same high affinity towards carbohydrates as the dimeric form, whereas the thermodynamic properties of this interaction are different in the two forms. In this sense ConA-m is a potentially useful tool in biological studies in order to explore the relation of the multiple valence of ConA molecule to the various induced effects in cell growth and metabolism (Nicolson 1976).

## Materials and methods

### *Preparation of Concanavalin A monomer*

Monomeric Concanavalin A (ConA-m) was prepared and purified according to the procedure reported by Tanaka et al. (1981), which was partially modified. Lyophilized ConA was purchased from Pharmacia Fine Chemicals. Appropriate amounts (about 200 ml) of 10 m*M* Tris Buffer, pH 7.3, in the presence of 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$, 10 m*M* methyl-α-D-mannopyranoside, 50 m*M* chloroacetamide and 1 *M* NaCl, were deoxygenated by bubbling through pure argon for 90 min and then used to dissolve the dry protein to a final concentration of 1 mg $ml^{-1}$. After 15 min of gentle bubbling and stirring, the protein

* To whom offprint requests should be sent

[1] Concanavalin A = ConA

[2] Monovalent photochemically alkylated monomer of Concanavalin A = ConA-m

solution was irradiated with a cylindrical high pressure UV lamp (150 W) for a further 90 min. The lamp was cooled with a solution of copper sulphate (2%) flowing in an appropriate hollow space (0.7 cm pass) made up of a cylindrical quartz vessel directly immersed in the solution to be irradiated and containing the UV lamp in the inner space. In this way the temperature in the solution vessel never exceeded 22 °C. The presence of copper sulphate in the cooling liquid absorbed radiation with wavelengths lower than 290 nm. The protein solution was then extensively dialysed against 0.1 *M* Tris Buffer, pH 7.3, in the presence of 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 0.1 *M* NaCl (TBS), in order to eliminate the sugar. Three irradiated batches were pooled and applied to a column of Sephadex G-100 (3.4–88 cm) equilibriated with TBS for affinity chromatography separation. The monomeric and tetrameric forms of the protein were separated and eluted with a linear gradient of D-Glucose (90 m*M* in 2,200 ml). The first peak from the column was characterized as the monovalent form of Concanavalin A. Two other major peaks were eluted, the last of which was identified as the tetramer (Tanaka et al. 1981). The first peak was concentrated by ultrafiltration (Amicon) to a concentration of 15 mg $ml^{-1}$ and then passed through a column of Bio Gel P-100 (3 × 150 cm) and eluted with TBS, in order to eliminate contaminations due to the dimeric and tetrameric forms still present. The purified fraction of ConA-m was then concentrated and rechromatographed with the same Bio Gel column, giving a single peak with no detectable impurities. Finally, ConA-m was concentrated and dialysed against 1 m*M* acetate buffer, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 4.5 and then used for the subsequent analysis and calorimetric experiments.

The yield of ConA-m with respect to the total amount of ConA used in each preparation was about 15%; several preparations were pooled into a single batch solution which was used in all the subsequent calorimetric binding studies.

Protein concentrations of ConA-m solutions were calculated by UV absorbance at 280 nm using an extinction coefficient of $E^{1\%}_{1cm} = 11.4$ (Brewer et al. 1973; Doyle et al. 1975), assuming a molecular weight of 27,000, as is discussed in detail in the next section. In the binding experiments the concentration of ConA-m was maintained constant whilst that of the ligands (sugars or glycoproteins) was varied.

*Preparation of immunoglobulins*

γ-Globulins (7S) from human sera were obtained from Calbiochem. After dialysis against phosphate-buffered saline (0.15 *M* NaCl, 0.02 *M* phosphate buffer, pH 7.2), the IgG suspension was purified by ion exchange chromatography on DEAE-Sephadex A 50 equilibrated with the same buffer (Fahey and Terry 1973). The unbound fraction was checked for purity by standard immunodiffusion and immunoelectrophoresis techniques using a commercial anti-gamma globulin serum (Ouchterlony and Nilssom 1973).

IgM (19S) was obtained by plasmapheresis from a patient with Waldenstrom's macroglobulinemia type *k* and purified as specified elsewhere (Dani et al. 1982). The purity of immunoglobulin preparations was checked by standard immunodiffusion and immunoelectrophoresis techniques using commercial antisera (Boehring). Immunoelectrophoresis with rabbit anti-human serum proteins showed a single band corresponding to IgM. Contaminants were detected with ultra-low level immunodiffusion plates. Only traces of albumin were seen and estimated to be lower than 5% of the total protein content.

Concentrations of IgM solutions were obtained by UV absorbance at 280 nm, corrected for light scattering according to the procedure of Leach and Scheraga (1960). The extinction coefficient used was $E^{1\%}_{1cm} = 13.5$, assuming a molecular mass of $8.9 \times 10^5$. Immunoprotein solutions were extensively dialysed against the same buffer used for ConA-m.

*Chemicals.* The sugars used in the binding experiments were grade A, chromatographically homogeneous and were purchased from Calbiochem. They were used without further purification and the solutions were made up by weighing the dry powder. All the chemicals employed were reagent grade.

*Calorimetry.* Calorimetric experiments were performed on an electrically calibrated LKB 10700 flow calorimeter (Monk and Wadso 1969). Two twin LKB peristaltic pumps operating at a flow rate of $3.1 \times 10^{-6}$ l · $s^{-1}$ were used for two independent solutions to be mixed in the reaction cell inside the calorimeter. After mixing the power signals were recorded. The enthalpy of dilution of each protein or sugar solution was independently measured and subtracted from the total enthalpy of reaction. No enthalpy of dilution for ConA-m in the concentration range studied was observed, and only small effects were detected for the most concentrated sugar solutions.

Power effects ranged from 0 to $30 \times 10^{-5}$ W. The measurements were made at 25 and 37 °C.

*HPLC measurements.* HPLC (High Performance Liquid Chromatography) experiments were carried out with a Waters HPLC instrument equipped with Protein Columns I-60, I-125 and I-250, equilibrated

overnight with TBS. The working pressure was kept at 3,000 psi.

*Amino-acid titration.* Fluorescence measurements were performed with a Perkin-Elmer LS3 photometer. Two solutions with identical optical densities (within instrumental uncertainty) were prepared with ConA-m and with the dimer in order to get exactly the same concentration for both on a subunit basis. The two solutions were then used for titration of the $-NH_2$ groups of the amino-acid residues following alkaline hydrolysis. The number of the amino-groups added by the alkylation reaction with chloroacetamide following UV irradiation (Hamada and Yonemitsu 1977) was considered negligible compared to the total number of $-NH_2$ groups present after hydrolysis. The amino-groups were titrated with fluorescamine according to Lay (1977) and a solution of bovine serum albumin (MW = 67,000) of known concentration was used as standard reference. A linear plot of fluorescence emission versus initial protein concentration allowed the best estimate of the concentration of ConA-m solutions, and, since the optical densities were known, of the extinction coefficient of the protein.

*Ultracentrifuge.* The sedimentation coefficient, *s*, was obtained at 5.7 °C with an analytical ultracentrifuge (Beckman Spinco Model E), equipped with a 12 mm single sector cell. The rotor speed was 56,000 rpm. Protein concentration was of the order of 3–4 mg/ml in acetate buffer.

*Binding studies.* The binding of methyl-α-D-[U-$^{14}$C]-glucopyranoside (3 μmCi/mmol, Amersham) was studied by equilibrium dialysis at room temperature (21 ± 0.5 °C). Small bags made of dry dialysis tube were partially filled with about two ml of protein solution at fixed concentration and extensively equilibrated with about 100 ml of sugar solutions at different concentrations. The dpm were counted with a LKB Wallac β-counter. The exact amounts of solution employed was determined by weighing the samples. The buffer used was 1 m*M* acetate, pH 4.5, with the same manganese and calcium concentrations as above.

*Agglutination tests.* Sheep red blood cells were brought to a concentration of 1% in Hanks' balanced salts solution, pH 7.2. 100 μl of red cell suspension was added to 100 μl of ConA solution in round bottom microtiter plates. Hemoagglutination was microscopically checked four hours later.

## Results

Figure 1a shows the elution chromatogram profile of the Sephadex G-100 column, where three major peaks were eluted as a function of a linear gradient of D-Glucose. The first peak has been identified as the monomeric form of ConA (Tanaka et al. 1981). Under our experimental conditions the total amount of ConA-m obtained did not seem to be dependent on the irradiation time; a longer exposure time gave a higher yield of low molecular weight fragments, probably due to the cleavage of the molecule.

Figure 1b shows a further step in the purification by gel filtration of the compound giving rise to the first peak in Fig. 1a on a column of Bio-Gel P-100.

The purified fraction of ConA-m was then concentrated and rechromatographed on the same Bio Gel column, giving a single peak with no detectable impurities. Moreover, purified ConA-m showed full binding activity towards carbohydrate

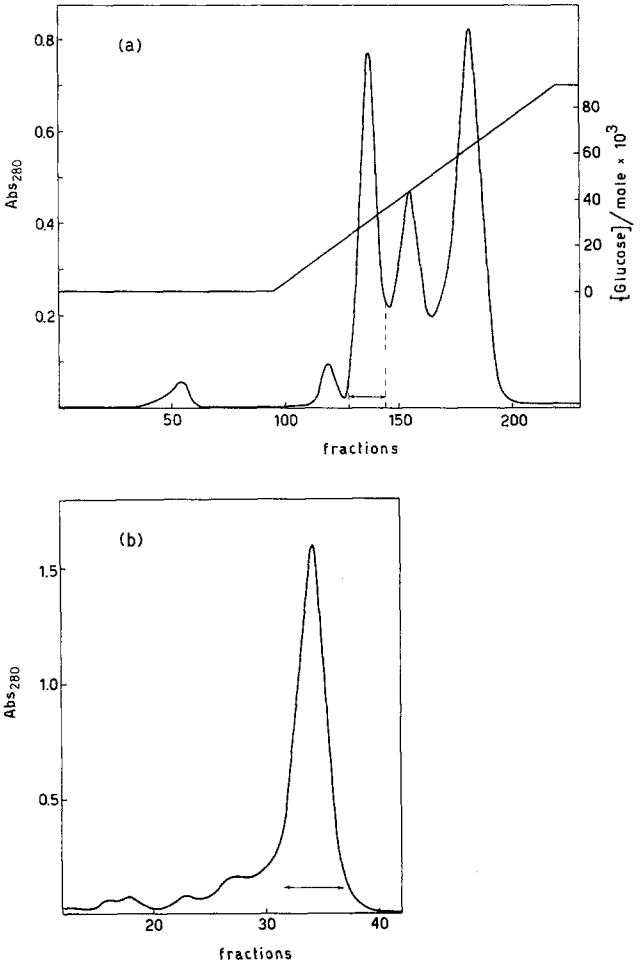


**Fig. 1. a** Elution chromatogram profile of photoalkylated Concanavalin A on a Sephadex G-100 column (3.4 × 88 cm). D-Glucose gradient 0–90 m*M* in 2,200 ml in $10^{-2}$ *M* tris buffer, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 7.3; 16 ml fractions were collected at a flow rate of 30 ml/h; **b** Bio-Gel P-100 gel filtration profile of the first peak eluted from Sephadex G-100 chromatography. 3 × 150 cm column using a flow rate of 30 ml/h. 9 ml fractions

substrates such as Sephadex G-100 when it was again passed through a column of this gel.

We did not study the other two major peaks detected by gradient chromatography. However, since the third and last peak consisted of about 50% of the total ConA, we tried to utilize this fraction to prepare more monomeric ConA. This fraction was then reirradiated under the same conditions as before but only a barely detectable amount of monomeric ConA-m was recovered, indicating that this form was no longer sensitive to alkylation and therefore was permanently modified by the first irradiation. The ConA present in the second peak was tentatively checked during the HPLC measurements described below; the results indicate that the form present in this peak has a molecular mass of about 24,000.

Since the chromatographic experiments described above showed that the subunit preparation was homogeneous we checked the biological activity of the molecule. ConA dimers and tetramers are able to agglutinate cells by forming cross links between polysaccharide side chains belonging to different cell membranes. This causes the formation of an extensive network of cells which leads to the agglutination. ConA-m should still be able to bind to the polysaccharide sides chains of a single cell but should not cause any agglutination. We therefore performed agglutination tests with sheep red blood cells comparing the activity of ConA-m with that of the dimeric form. No agglutination was observed at monomer concentrations up to 3–4 mg/ml, whilst the dimer showed an agglutination activity which disappeared after diluting a 5–6 mg · ml$^{-1}$ sample about 128 times. Therefore the presence of tetrameric contaminants was estimated to be less than 1%.

The monomeric subunit purified in this way should correspond to a molecular mass of 27,000, half that of the dimer which has been estimated (Edelman et al. 1972; McKenzie et al. 1973; Wang et al. 1971) to be 54,000 at pH 5 or below. The results of our purification agree with those reported in the literature (Tanaka et al. 1981), nevertheless further physico-chemical characterizations were performed.

Molecular weight was determined by SDS (1%) polyacrylamide gel electrophoresis. Dimeric ConA dissociates into monomeric subunits in the presence of SDS. Four bands are observed: the larger one corresponds to a molecular weight of 29,400, the others to molecular weights of 27,000, 14,000 and 13,500, respectively. These last three bands demonstrate the heterogeneity of conventionally prepared ConA solutions, which is due to the presence of naturally occuring low-molecular weight fragments (Cunningham et al. 1972; Sitrin et al. 1982; Williams et al. 1978). ConA-m showed three bands:

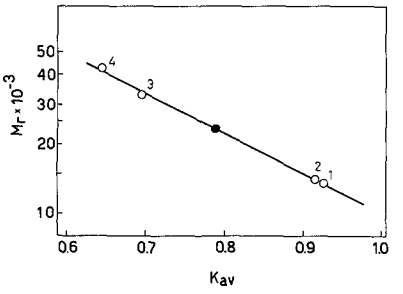


**Fig. 2.** Calibration curve of molecular size obtained by HPLC. $K_{av}$ values plotted as a function of log $M_R$ (psi 3,000). 1) ribonuclease; 2) lysozyme; 3) superoxydedismutase; 4) ovalbumin. Columns: Waters I-60, I-125 and I-250

a major one corresponding to a MW of 29,300 and two faint bands, estimated to be less than 8% of total amount, which corresponded to the intermediate molecular weights. The lowest molecular weight band seen after electrophoresis of the dimeric ConA completely disappeared. The affinity chromatography on Sephadex G-100 was therefore sufficient to prepare purified ConA-m, free of the naturally occurring fragments, as indicated by Williams et al. (1978) for the ConA-dimers. In order to confirm that the alkylated ConA was a monomer and not a dimer, its molecular size was also checked by exclusion HPLC. The elution volume factor, $V_e$, was recorded using both the UV absorption at 254 nm and the refractive index; both gave identical results. Figure 2 shows the calibration curve which is constructed by plotting $V_e$ as a function of the logarithm of the molecular weight. ConA-m gave a single wide peak under our experimental conditions, whose $V_e$ corresponds to a hydrodynamic mass of 23,000, as calculated by linear interpolation of the calibration curve.

The ultracentrifuge experiments gave a sedimentation coefficient of $s_{20} = 2.6$ Svedberg and a single symmetric boundary was observed. A value of $s_{20} = 4.0$ was found for the dimer in our buffer conditions, confirming the results previously reported (Toselli et al. 1981).

The results obtained by fluorescence titration of ConA-m tentatively indicate that the extinction coefficients of the monomeric and dimeric protein do not differ significantly. The value found for ConA-m was $E^{1\%}_{280} = 11.15 \pm 0.03$.

*Reaction of ConA-m with sugars.* A Scatchard plot of the results of the equilibrium dialysis binding experiments of ConA-m and the radioactive methyl-α-D-glucopyranoside is shown in Fig. 3. the value of the observed association constant is $3.15 \pm 0.15 \times 10^3$ mol$^{-1}$ and the number of the binding sites $0.998 \pm 0.05$, based on a molecular weight of 27,000.

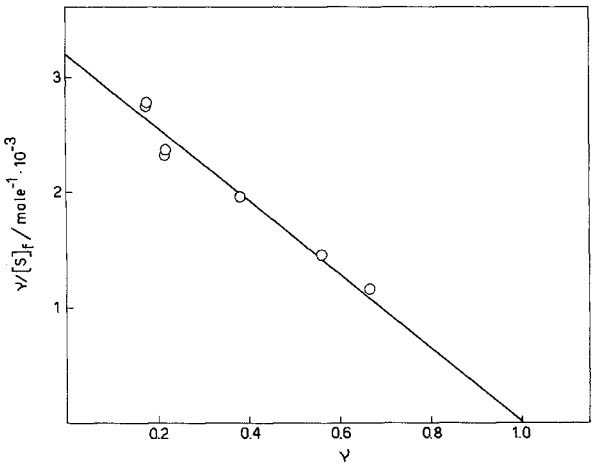


**Fig. 3.** Scatchard plot of equilibrium dialysis data for the binding of methyl-α-D-[U]$^{14}$glucopyranoside to monomeric photoalkylated concanavalin A. Measurements were carried out at 21 °C and a pH of 4.5

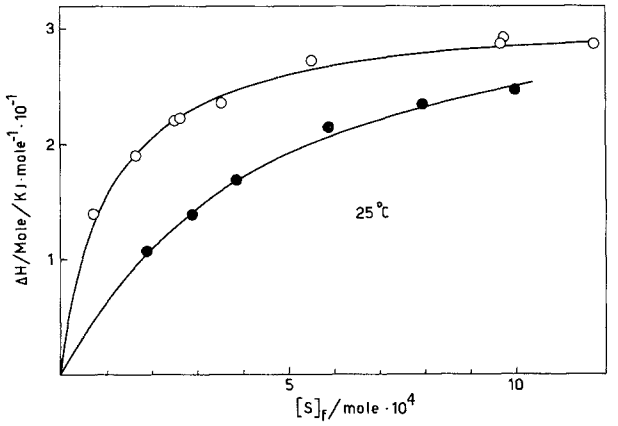


**Fig. 4.** Thermal titration of monomeric photoalkylated concanavalin A with methyl-α-D-mannopyranoside in: (●) 1 m*M* acetate buffer, (○) $10^{-1}$ *M* acetate buffer; and 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 4.5. The solid lines represent the calculated binding curves obtained by least square treatment according Eq. (1)

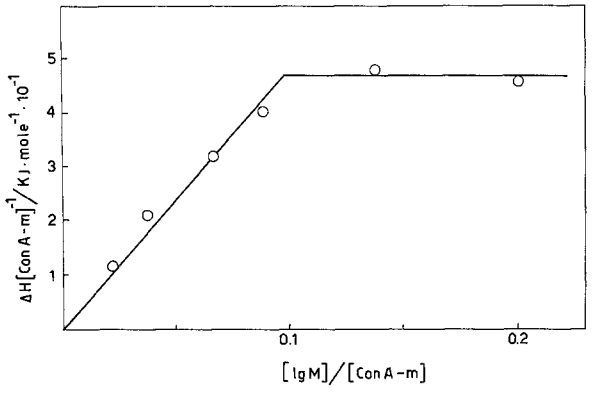


**Fig. 5.** Thermal titration of monomeric photoalkylated concanavalin A with IgM (19S). Heat effects are plotted in function of the ratio of protein molar concentrations. Experiments were carried out at 25 °C

Using the calorimetric $\Delta H$ (this work), it is possible to calculate the $K_a$ at the temperature of the equilibrium dialysis experiment: the value obtained at 21 °C is $3.6 \pm 0.15 \times 10^3$ mol$^{-1}$. This calculated constant has a large uncertainty, but it might also imply a non-linear relationship between $\ln K_a$ and the reciprocal of the temperature.

The enthalpies of the binding reaction between ConA-m and some pyranosides were measured calorimetrically at 37 and 25 °C, at pH 4.5. The total heat evolved was considered to be the result of the binding reaction and therefore correlated with the sugar added. The experimental heats of reaction, $Q_{exp}$, with methyl-α-D-manno, methyl-α-D-gluco and p-nitrophenyl-α-D-mannopyranoside as a function of the concentrations of free sugar have been investigated and in Fig. 4 some examples are reported. The values of $\Delta H$ ($Q_{max}$) and of the association equilibrium constant, $K_a$, can be calculated by an iterative least-square treatment of the following equation according to Bolen et al. (1971) and applied by Dani et al. (1981) to the dimer system.

$$\frac{1}{Q_{exp}} = \frac{1}{\Delta H} + \frac{1}{\Delta H K_a [S]_f}, \qquad (1)$$

where $[S]_f$ is the concentration of the free pyranoside derivative. The results and the calculated thermodynamic quantities of the reactions are summarized in Table 1. The indicated errors are those obtained by least-squares best fitting of the experimental data. The van't Hoff $\Delta H$ values calculated from the temperature dependence of the equilibrium constant agree well with the calorimetric values.

*Reaction of ConA-m with IgG (7S).* No heat of reaction was recorded after mixing of the two solutions of macromolecules. This confirms that the enthalpic contribution to $Q_{exp}$ of this reaction, if any, is negligible (Dani et al. 1982).

*Reaction of ConA-m with IgM (19S).* The measured enthalpies of reaction of the two macromolecules as a function of their molar concentration ratio are plotted in Fig. 5. The experiments gave a $\Delta H$ value of $-47.5 \pm 1$ KJ · mol$^{-1}$ of ConA-m. The enthalpy apparently becomes constant for ratios greater than 0.1, indicating an apparent stoichiometry of 1 IgM mole to $10 \pm 0.5$ moles of ConA-m. Since the pentameric IgM molecule has two heavy chains per subunit, the stoichiometry corresponds to one ConA-m molecule per heavy chain. This reaction was studied only at 37 °C since an identical reaction with the dimer has been reported to be unaffected by changes in temperature (Dani et al. 1982).

**Table 1.** Thermodynamic parameters of the reaction between Concanavalin A and monomeric photoalkylated Concanavalin A with pyranosides and immunoproteins at pH 4.5. Listed error is the standard deviation of least-squares treatment according to Eq. (1)

| | $\Delta H$ [kJ/mol] | $\Delta G$ [kJ/mol] | $\Delta S$ [e. u.] | $K$ [$mol^{-1} \times 10^3$] | $\Delta Cp$ [J/mol $K$] | $I$ [mol] | $T$ [°C] |
|---|---|---|---|---|---|---|---|
| | | | ConA | | | | |
| methyl-α-D-mannopyranoside[a] | −22.8 ± 0.2 | −22.8 ± 0.1 | 0.0 ± 0.3 | 8.6 ± 0.6 | – | $10^{-1}$ | 30 |
| [c] | −23.8 ± 0.6 | | | | | 1 | 25 |
| methyl-α-D-mannopyranoside[a] | −18.8 ± 0.3 | −22.9 ± 0.4 | 18.7 ± 0.3 | 9.1 ± 1.5 | – | $10^{-3}$ | 30 |
| methyl-α-D-mannopyranoside | −21.7 ± 0.2 | −25.9 ± 0.4 | 13.3 ± 0.3 | 22.8 ± 1.5 | −540 ± 100 | $10^{-3}$ | 37 |
| [c] | −24.7 ± 0.6 | | | | | $10^{-3}$ | 25 |
| methyl-α-D-glucopyranoside[a] | −8.3 ± 0.1 | −22.0 ± 0.2 | 44.0 ± 0.9 | 5.1 ± 0.1 | −260 ± 100 | $10^{-3}$ | 37 |
| pN phenyl-α-D-mannopyranoside[a] | −13.9 ± 0.9 | −27.3 ± 0.2 | 13.8 ± 0.4 | 40.0 ± 0.6 | −140 ± 100 | $10^{-3}$ | 37 |
| IgG | 0 | – | – | | | $10^{-3}$ | 25–37 |
| IgM[b] | −24.0 ± 0.5 | – | – | $>10^3$ | 0 | $10^{-3}$ | 25–37 |
| | | | ConA-m | | | | |
| methyl-α-D-mannopyranoside | −31.6 ± 0.2 | −22.7 ± 0.3 | −29.8 ± 0.3 | 9.6 ± 0.7 | – | $10^{-1}$ | 25 |
| methyl-α-D-mannopyranoside | −35.9 ± 0.1 | −19.2 ± 0.4 | −55.8 ± 0.4 | 2.3 ± 0.5 | −600 ± 150 | $10^{-3}$ | 25 |
| methyl-α-D-mannopyranoside | −43.6 ± 0.1 | −21.9 ± 0.4 | −69.8 ± 0.4 | 4.9 ± 0.5 | – | $10^{-3}$ | 37 |
| pN phenyl-α-D-mannopyranoside | −48.6 ± 0.1 | −21.8 ± 0.4 | −87.9 ± 0.5 | 4.9 ± 0.6 | – | $10^{-3}$ | 37 |
| methyl-α-D-glucopyranoside | −33.4 ± 0.5 | −19.5 ± 0.4 | −45.0 ± 0.4 | 1.9 ± 0.6 | – | $10^{-3}$ | 37 |
| IgG | 0. | | | | | $10^{-3}$ | 37 |
| IgM | −47.0 ± 0.5 | – | – | $>10^3$ | – | $10^{-3}$ | 37 |

[a] Ref. Dani et al. 1981
[b] Ref. Dani et al. 1982
[c] Ref. Munske et al. 1984 (at pH 4.3, MW = 25,500, $E_{280} = 1.37$ ml/mg)

## Discussion

The electrophoretic results presented here indicate that ConA-m is highly purified compared to the dimer. We have assumed the molecular weight of ConA-m to be 27,000, half of the reported value of the dimer (Edelman et al. 1972; McKenzie et al. 1973, Wang et al. 1971). This assumption correlates with the decrease of the sedimentation coefficient of ConA-m compared with the dimer. The single peak observed on the analytical ultracentrifuge confirms the homogeneity of the preparation. A single symmetrical peak was also obtained by the HPLC technique, which gave a somewhat lower value for the molecular mass, probably due to the shape of the molecule and its interactions with the specific stationary phase of the Waters columns.

According to Hamada and Yonemitsu (1977), after UV irradiation at least one tryptophan residue is alkylated per subunit of ConA. This event may be responsible for the conversion of the tetramer into monomer. By inspection of the crystallographic data (Reeke et al. 1978) the modified residue may be either TRP 88, involved in the monomer-monomer interaction in the tetrameric molecule, or TRP 182 which is exposed to the solvent. The alkylation has not been responsible for changes in the extinction coefficient of the protein.

Scatchard plot analyses of the equilibrium dialysis binding experiments confirms the monovalent character of the molecule. Moreover, the equilibrium constant for association of methyl-α-D-glucopyranoside with ConA-m is in reasonable agreement with the calorimetric data for the same reaction shown in Table 1, but differs from that observed for the dimer reacting with the same sugar (Dani et al. 1981). Using the $\Delta H$ for the reaction of the dimer with methyl-α-glucopyranoside, the value of $K_a$ at 21 °C is $6.5 \times 10^3$ $mol^{-1}$ instead of $3.6 \times 10^3$ as for the monomer (Table 1).

The sign and magnitude of $\Delta H$'s for the various derivatives indicate $H$-bonding and van der Waals interactions as exemplified by the reaction with methyl-α-D-mannoside. The sign of $\Delta S$ in the ConA-m system is negative in all the cases studied, whereas it becomes positive in the dimer reactions. The magnitudes of these quantities are the net result of different and opposite contributions, which include negative contributions due to alterations of the degree of freedom of both molecules upon binding (Eftink and Biltonen 1980), positive contributions due to the release, upon binding, of water molecules interacting with the sugar (Tait et al. 1972) and perturbation of the solvent molecules (Eisenberg and Kauzmann 1969; Lumry et al. 1982). These phenomena may give different net contributions in the two ConA systems.

It is interesting to note that the structure of the methyl-α-D-glucopyranoside molecule differs from that of methyl-α-D-mannoside because the −OH

group of carbon 2 has an equatorial rather than an axial orientation. This is enough to decrease the $\Delta H$ for binding of the methyl-α-D-glucoside to ConA-m by about 10 KJ · mol$^{-1}$ which is about the magnitude expected for a *H*-bonding interaction. The same behavior was observed for the dimer.

The sign and magnitude of $\Delta H$'s and $\Delta S$'s in Table 1, suggest that the binding of the methyl-α-D-manno and -gluco derivatives to ConA-m has a hydrophilic-like character. Surprisingly, the reaction with *p*-nitrophenyl-α-D-mannopyranoside has the same characteristics, even in presence of a large and relatively hydrophobic residue: the value of $\Delta S$, instead of being less negative, as expected for a more pronounced hydrophobic interaction, becomes even more negative as does the enthalpy change. The binding site still 'sees' the sugar as a nonhydrophobic ligand with which it is possible to make strong hydrophilic contacts. Table 1 also reports the calculated value of $\Delta C_p$ for the reaction of ConA-m with methyl-α-D-mannoside. As reviewed by Eftink and Biltonen (1980), a decrease in the heat capacity in a binding reaction may be regarded as a change in the distribution of microstates available to the protein with respect to its complex by decreasing the probability that the macromolecule exists in the higher enthalpy (or entropy) states of its energy distributions (Cooper 1976). These are more closely related to low rotational and vibrational frequency modes of the protein. The ligand tethers different regions of the molecule, altering the interactions among atoms and their cooperative motions, lowering the average enthalpy value of the system. In view of these arguments, subtle conformational rearrangements (Lumry and Biltonen 1969; Lumry and Rosenberg 1976) may give the observed decrease in the heat capacity in our system. ConA-m, therefore, may be envisaged as a "looser" molecule than its complex with sugar. The ligand ties up the protein with binding interactions whose influence are felt at the binding site level as well in the surrounding molecular domains.

It is interesting to note that specific saccharides inhibit the denaturation of monomeric fragments of ConA (Cunningham et al. 1972). After binding the sugar, the monovalent ConA fragments increase their stability towards denaturation. Negative $\Delta C_p$'s are also observed for the dimer system, but the values are less negative. Apparently, it is possible to consider the dimer as a more rigid molecule, which is not greatly perturbed on ligation, whereas the two monomers, after their assembly, have less ability to fluctuate (Senear and Teller 1981). This hypothesis could also be inferred from X-ray diffraction studies (Reeke et al. 1975), which show that the entire back of the ellipsoidal dimer consists of a single 12-stranded antiparallel pleated sheet, with half of its chains contributed by each protomer. This is a highly organized structure, which should stabilize monomer-monomer interactions. In fact, the dimer dissociation has only been tentatively reported (Fraser et al. 1976; Sophianopoulos and Sophianopoulos 1982).

As shown in Table 1, for the dimer system the values of $\Delta H$ for all pyranosides are negative but much lower compared to the monomer (about 20 KJ · mol$^{-1}$), and the values of $\Delta S$, become positive. Moreover, the values of $\Delta G$ do not differ significantly from those of the monomer system for the reaction with the same sugar, the affinity being slightly higher. The hydrophobic and hydrophilic-like contributions to $\Delta H$ and $\Delta S$ cannot be unambiguously separated, but their relative importance may change in the two ConA systems, giving, as a net result, changes in the sign and magnitude of the thermodynamic parameters of binding. This may imply a different binding mechanism for the two forms of ConA. For instance, the dimer binds *p*-nitro-phenyl-mannose with a slightly lower $\Delta S$ and lower $\Delta H$ than the methyl-manno derivative, as expected for a more 'hydrophobic'-like character of the reaction, while ConA-m shows the opposite behavior.

In Table 1 the results at high ionic strength are reported for the reaction of the methyl-α-D-mannoside derivative with both monomeric and dimeric ConA. The presence of salts does not alter the affinity of the ConA towards the sugar, while the change in entropy for the reaction goes to zero from a positive value. This might be consistent with the thermodynamic change accompanying the release of water molecules bound either to the binding site and/or interacting with the sugar moiety after the binding reaction. If the number of water molecules released to the solvent is the same as in the absence of salts, the presence of salts makes the aqueous solvent more structured-like (Jolicoeur and Desnoyers 1983) and therefore the entropy change for the reaction should be less positive.

ConA-m shows different binding behavior in the presence of salts. The affinity towards methyl-α-D-mannoside increases, while the entropy change becames less negative. This observed difference is also reflected by the $\Delta H$ changes for the same reaction, which are similar in magnitude but opposite in sign. It has been reported (Pflumm et al. 1971; Grimaldi and Sykes 1975; Bailey et al. 1978) that the binding of the sugar molecule itself induces a conformational change in the ConA dimer, but this does not seem to make any major contribution to the thermodynamic quantities for binding (Dani et al. 1981). It is conceivable that this conformational

rearrangement may also be involved with ConA-m binding reactions.

The different behavior of ConA-m and the dimer implies that the monomeric subunits should undergo a conformational change upon assembly, if our monomeric species is equivalent to the dimeric unchanged subunit. There should be an interaction energy between the events occurring at the binding site and the monomer-monomer interface: the monomer needs an average 20 KJ/mol more than the dimer to bind sugars in order to overcome the unfavourable entropy of binding, which is, in turn, used up upon assembly. Linkage systems involving association equilibria have been reported for ConA (Senear and Teller 1981) and for other protein (Wiesinger et al. 1979). In our system, a linkage energy between the dissociation and the binding functions is probably present, but not between the two binding sites of the dimer: they remain independent and non-cooperative upon assembly of the two monomers (Christie et al. 1978; Dani et al. 1981). This behavior has also been observed in other systems (White 1975).

ConA-m is able to bind IgM with the same stoichiometry as the dimer (Dani et al. 1982). If we assume that mannose is the most frequent terminal sugar of IgM saccharides chains (Johannean and Bourillon 1976), the data indicate that ConA-m reacts with the IgM subunit with an enthalpic contribution very close to the value of the methyl-α-D-mannoside (Fig. 5). It does seem that ConA binds free monosaccharides in the same way as glycoproteins, because even when a conformational change may be required to justify the binding properties of monomeric and dimeric forms reacting with free monosaccharides, the same conformational rearrangements are preserved and required for the binding of ConA molecule to the saccharide chains of IgM. As discussed by Dani et al. (1982) steric constraints may be responsible for the 1 : 10 stoichiometry of the reaction of the dimer with the pentameric IgM molecules. Because each subunit has two heavy chains, only one site of the bivalent dimeric ConA per heavy chain has the correct spatial orientation for reacting with the terminal sugar of only one of the saccharide chains present in each heavy chain. The same steric constraints are still present even when the size of the ConA molecule is halved.

In conclusion, we found that ConA-m has a binding affinity towards monosaccharides similar to that of the dimeric form, but it differs from the latter on the basis of the enthalpy and entropy changes of the binding reaction. These differences may be related to the energetic mechanism of these interactions which are different for the two forms: ConA dimer appears to react as a compact molecule, whereas ConA-m reacts as a more 'loose' molecule. Moreover ConA-m still shows a high affinity towards glycoproteins as is the case for the dimeric form.

Cell recognition, in conditions where no agglutination is desired, and specific labelling of cell receptors without steric hindrance incompatibility, appear as possible biological applications for the monomer ConA derivative.

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