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STABILIZATION OF CONCANAVALIN A BY METAL LIGANDS

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ABSTRACT

Metal-free concanavalin A is readily and irreversibly inactivated by temperatures above 60°. Manganese ion completely prevents the thermal aggregation of the protein at 60 and 70°, and partially protects at 80°, but shows no protective properties at 90°. Manganese protection against thermal aggregation was found to be maximal at pH 4–8. The precipitation between glycogen and Mn^{2+}-stabilized concanavalin A is partially inhibited at temperatures >30°, but can be reversed by cooling to room temperature. Manganese ion also partially reverses the inhibition of concanavalin A–glycogen complex-formation by protein denaturants and by saccharides. At 20°, calcium ion plus Mn^{2+} prevents the aggregation of metal-free concanavalin A induced by guanidine hydrochloride. The results show, contrary to conclusions from previous studies, that concanavalin A is a highly stable protein when supplemented with Mn^{2+} and Ca^{2+}.

INTRODUCTION

Several articles have appeared showing that concanavalin A (con A), the jack-bean phytohemagglutinin or lectin, has marginal stability in solution. So and Goldstein[1], McKenzie *et al.*[2], and Cunningham *et al.*[3] noted that con A solutions tend to aggregate spontaneously. Doyle *et al.*[4] found that temperatures above 50° enhance the aggregation of con A, but that the aggregation can be partially prevented by specific sugars that bind to the protein. Pflumm and Beychok[5] recently found that denaturants, such as urea, irreversibly inactivate con A. Earlier, Goldstein *et al.*[6] had shown that pH values >9.0 result in the loss of carbohydrate binding by con A. Thus, one of the inherent difficulties in working with con A appears to be its ease of denaturation and its nonspecific aggregation.

In studies on the crystal structure of con A, Hardman[7] predicted that the protein's native metals, Mn^{2+} and Ca^{2+}, would impart stability to the molecule. By use of several techniques, we now find that manganese and calcium ions confer a high degree of structural stability to native con A, as well as to metal-free con A.

EXPERIMENTAL

Materials and methods. — Native con A was prepared from defatted jack-bean meal (Schwarz/Mann, Orangeburg, N.Y.) by Sephadex adsorption according to Agrawal and Goldstein[8]. Metal-free con A was prepared by dialyzing native con A against cold 0.1M hydrochloric acid, pH 1.5–2.0, containing 0.5M sodium chloride[9]. Metals in the protein preparations were determined by atomic absorption, using an Instrumentation Laboratory, Inc. Model 253 atomic absorption spectrophotometer. Native con A contained 0.75 mole of Mn^{2+} and 0.60 mole of Ca^{2+} per mole of con A subunit (mol. wt. 27,000). Metal-free con A contained 12 mmoles of Mn^{2+} and 160 mmoles of Ca^{2+} per mole of con A subunit (see also ref. 10). Concentrations of con A were determined spectrophotometrically, by assuming that 1 mg/ml gives an absorbance of 1.14 at 280 nm with a 1-cm path-length[11].

Methods for monitoring the thermal aggregation of con A have been described[4]. The following buffers were used to study the effect of pH on the thermal aggregation of con A: pH 2.0, hydrochloric acid–potassium chloride; pH 3.0, potassium phthalate; pH 4.0–6.0, acetate; pH 7.0, 2-amino-2-(hydroxymethyl)-1,3-propanediol maleate (Tris maleate); pH 8.0–9.0, Tris; and pH 10, glycine–sodium hydroxide. The final buffer concentrations were all 0.05M.

The precipitation between glycogen and con A was monitored turbidimetrically[12] at 600 nm by use of a Bausch & Lomb Spectronic 20 instrument with cylindrical, 1.25-cm cuvets. Conditions for the interactions are given in the Discussion. Inhibition studies were also performed turbidimetrically.

All salts were reagent grade. Guanidine hydrochloride (GuCl) "Ultrapure" was purchased from Schwarz/Mann, Orangeburg, N.Y., and rabbit-liver glycogen from Sigma, St. Louis, Mo.

Sedimentation studies were conducted with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. All sedimentation-velocity experiments were made at 59,780 rev·min^{-1} at 20°. A partial specific volume of 0.73 ml/g was used in the calculations. Sedimentation coefficients were corrected for the density and viscosity of the solvent by using the Svedberg–Pedersen equation[13,14].

RESULTS AND DISCUSSION

Metal-free con A readily forms visible aggregates when heated at 60–80° (see Fig. 1). Furthermore, the addition of Mn^{2+} to the protein solution results in a marked decrease in the rate of aggregation. Incubation of metal-free con A overnight at 60° in the presence of 1mM Mn^{2+} resulted in the loss of only 10 per cent of the protein due to aggregate formation. In the absence of transition metal, more than 90 per cent of the protein became insoluble within 60 min. Higher temperatures increased the rate of aggregation of metal-free con A (see Fig. 2). Complete protection by Mn^{2+} was observed at 70°; at 80°, only partial protection was found (see Fig. 2). The addition of Ca^{2+} had no effect on the thermal aggregation of con A, regardless

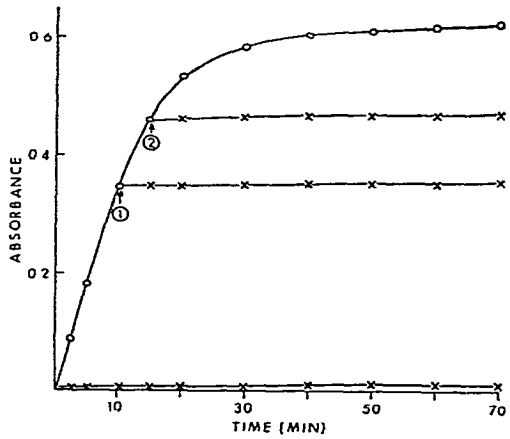

Fig. 1. Effect of manganese ion on the thermal aggregation of metal-free concanavalin A. [Metal-free con A (0.6 mg) in 0.05M sodium acetate–0.5M sodium chloride, pH 5.0, was incubated at 60°. Con A, —○—; con A+1mM $MnCl_2$, —×—. At the arrows, 1mM Mn^{2+} was added to the con A. The final volumes were 4.1 ml.]

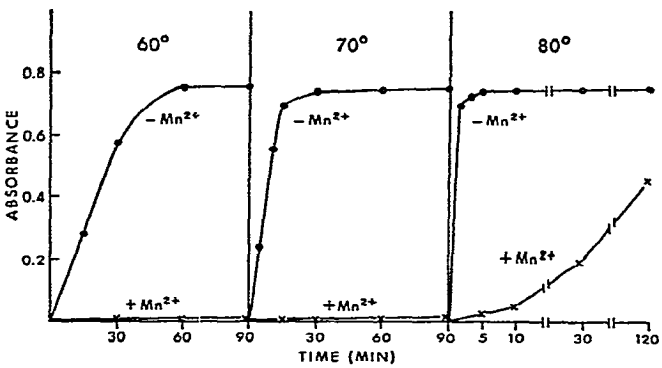

Fig. 2. Thermal aggregation of metal-free con A as a function of temperature. [The turbidities were determined in cuvets containing 0.5 mg of metal-free con A in 0.05M sodium acetate (pH 5.0) containing 0.5M sodium chloride. The final volumes were 4.1 ml. Manganese was added immediately prior to incubation at the chosen temperatures.]

of the presence of Mn^{2+}. Native con A is also readily inactivated at 60°, but at a lower rate[4]. No protection was detected at 90°. Subsequent experiments were performed at 60° in order to monitor the thermal aggregation of metal-free con A accurately.

In Fig. 3, the effect of pH on the thermal aggregation of con A is shown. Manganese ion was found to protect against aggregate formation between pH 4 and 8. Several workers have reported that con A binds carbohydrate ligands well within the same approximate pH range. At the higher pH values, protection is probably lost due to protein denaturation[5]. At lower pH values (2–3), Mn^{2+} does not bind to con A,

presumably because the side-chain carboxyl groups which coordinate to the metal are positively charged[7].

Native con A readily forms insoluble complexes with glycogen between pH 5 and 8 (ref. 1). Such protein denaturants as GuCl and potassium thiocyanate (KSCN) prevent the interaction either by interfering with hydrogen-bond formation or by changing the structure of the protein[15]. We find that by supplementing native con A with Mn^{2+}, inhibition of the precipitin reaction by chaotropic agents is partially prevented (see Fig. 4). For example, in the presence of KSCN, 50 per cent inhibition of the con A–glycogen reaction required an ~0.3 molar concentration of the inhibitor.

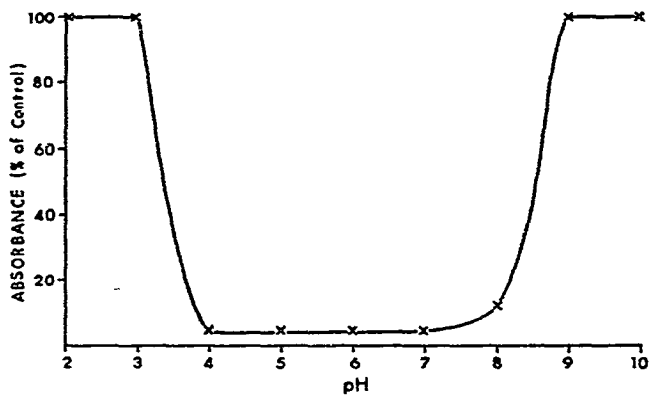

Fig. 3. Influence of hydrogen ion on the thermal aggregation of metal-free con A in the presence of Mn^{2+}. [Solutions of metal-free con A (0.6 mg) were prepared in the appropriate buffers in the presence and absence of 1mM Mn^{2+}. The final volumes were 4.1 ml.]

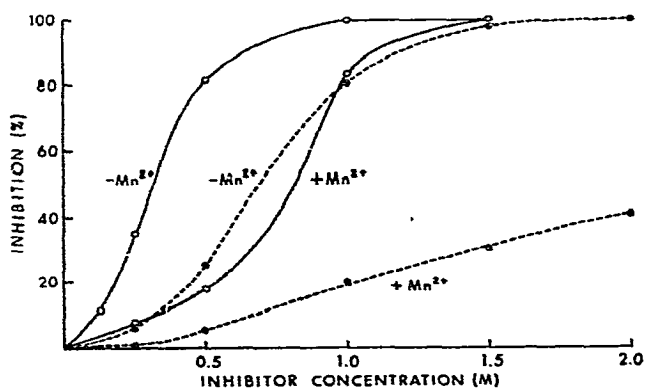

Fig. 4. Inhibition of con A–glycogen precipitation by guanidine hydrochloride and potassium thiocyanate. [All reaction-mixtures contained 0.5 mg of glycogen and 2.0 mg of native con A in a final volume of 4.1 ml. The solvent was 0.1M sodium acetate–0.5M sodium chloride, pH 5.6. Mn^{2+} was supplemented at a concentration of 1 mM. The inhibitors were KSCN, —O—; and GuCl --●--.]

When Mn^{2+} was added, ~0.8M KSCN was required for 50 per cent inhibition. In addition, Mn^{2+} also markedly lessened the inhibition by GuCl. Thus, supplementation of native con A with a transition metal confers significant resistance to con A in terms of inhibition by protein denaturants. The addition of Ca^{2+} afforded no increase in resistance to inhibition by GuCl or by KSCN.

We measured the inhibition of glycogen–con A precipitation by methyl α-D-mannopyranoside and by D-glucose in the presence and absence of added Mn^{2+} and Ca^{2+}. The results (see Fig. 5) showed that Mn^{2+} protects against inhibition by each saccharide. For example, in the absence of supplemented Mn^{2+}, 0.17 μmole

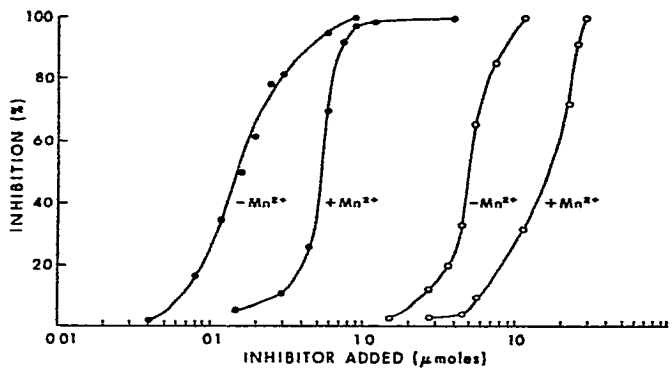

Fig. 5. Saccharide inhibition of concanavalin A–glycogen precipitation in the presence and absence of Mn^{2+}. [All reaction mixtures contained 0.5 mg of glycogen and 2.0 mg of native con A in 4.0 ml of 0.5M sodium chloride–0.1M sodium acetate, pH 5.6, plus the indicated amount of methyl α-D-mannopyranoside (—●—) or D-glucose (—○—). Mn^{2+}, when present, was added to a 1mM concentration.]

of methyl α-D-mannopyranoside gave a 50 per cent inhibition. When Mn^{2+} was present, 0.53 μmole of the saccharide was required for 50 per cent inhibition. Qualitatively similar results were obtained when D-glucose was used as the inhibitor (see Fig. 5). When Ca^{2+} was added to either native con A or to con A supplemented with Mn^{2+}, no detectable changes in resistance to inhibition by saccharides were observed. The foregoing results indicate that saturation of con A with Mn^{2+} enhances the protein's interaction with glycogen. Furthermore, the results suggest that the con A–glycogen interaction is more dependent on the presence of an excess of Mn^{2+} than on the presence of an excess of Ca^{2+}; this is probably due to the fact that con A binds Ca^{2+} more strongly than it binds Mn^{2+} (refs. 9 and 10).

It has been shown that 8.0M urea will not completely dissociate con A into its subunits. Olson and Liener[16] observed that aggregates of molecular weight >200,000 are formed in the presence of concentrated urea solution. We obtained similar results, as determined by sedimentation rates, for metal-free con A in the presence of guanidine hydrochloride (see Table I). In 3.0M GuCl, the sedimentation coefficient for metal-free con A was 15.30 S. In the presence of Mn^{2+} or Ca^{2+}, polymerization

still occurred. However, when Ca^{2+} was added to Mn^{2+}–con A, the sedimentation rate for the protein was normal (see Table I). Thus, metals effectively stabilize the protein in 3.0M GuCl in terms of protein–protein interactions. Brewer *et al.*[17]

TABLE I

SEDIMENTATION OF CONCANAVALIN A IN THE PRESENCE AND ABSENCE OF GUANIDINE HYDROCHLORIDE AND METAL LIGANDS[a]

GuCl (molarity)	*Metal added*	$S_{20,w} \times 10^{13}$
0.0	none	3.43
0.0	Mn^{2+}, Ca^{2+}	3.52
1.0	none	3.57
1.0	Mn^{2+}, Ca^{2+}	3.62
3.0	none	13.54
3.0	Mn^{2+}	15.30
3.0	Ca^{2+}	13.39
3.0	Mn^{2+}, Ca^{2+}	3.51

[a]Metal-free, intact subunit of concanavalin A was used in all centrifuge experiments. The concentration of protein for each experiment was ~8.0 $mg \cdot ml^{-1}$. Metals, where present, were added, at a 10^3 molar excess, 30 min prior to subjecting the protein to guanidine hydrochloride. All solutions were buffered with 0.1M sodium acetate, pH 5.6.

recently presented evidence that the Ca^{2+} ion serves to accelerate the rate of binding of Mn^{2+} to con A. X-Ray crystallographic data, however, clearly show that Ca^{2+} binds to the protein. Our results suggest that Mn^{2+} binds quickly to metal-free con A, without the need for Ca^{2+}. The addition of Mn^{2+} to metal-free con A results in the immediate cessation of thermal aggregation (see Fig. 1). We have recently been able to titrate con A with Ca^{2+} by use of ultraviolet spectral-difference measurements[10]. The difference spectrum induced by Mn^{2+} in metal-free con A is also generated immediately upon mixing the metal ion with the protein.

Because Mn^{2+} protects con A from thermal aggregation (see Figs. 1 and 2), examination of the effect of temperature on the con A–polysaccharide interaction should be possible without significant background turbidity due to the protein alone. From Fig. 6, it may be seen that, as the temperature is raised, a decrease in the extent of con A–glycogen complex-formation occurs. When the cuvets were cooled rapidly to 22° (as shown by the arrows), precipitates appeared. This effect was completely reversible, as several heating–cooling cycles could be performed with the same sample without alteration of the final turbidities. Methyl α-D-mannopyranoside (20 μmoles) completely inhibited any precipitin reaction, showing that the turbidities observed were caused by true con A–glycogen complexes. It is suggested that the inhibition noted at elevated temperatures is not due to protein denaturation (as the inhibition is readily reversed by cooling) but to prevention of formation of stable hydrogen-bonds between the protein and the polysaccharide; this concept is consistent with earlier observations[15].

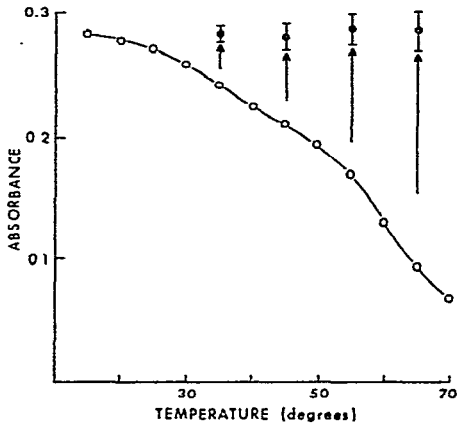

Fig. 6. Effect of temperature on the interaction between con A and glycogen. [Reaction mixtures contained native con A (1.7 mg) and glycogen (1.0 mg) in 1mM Mn^{2+}–0.1M sodium acetate–0.5M sodium chloride (pH 5.0). The final volumes were 4.0 ml. The con A and glycogen were mixed in cuvets, and held at 22° until the absorbance at 600 nm was constant. The cuvets were then placed in water baths at the temperatures indicated. After 5 min of incubation at the temperatures designated, the cuvets were withdrawn, and the absorbances immediately determined. The arrows indicate that the samples were transferred back to a bath at 22° and held therein for 20 min. The bars represent the extrema of 4 to 8 separate samples at the given temperature; —●— represents the mean of these absorbances.]

We have shown that the addition of Mn^{2+} to metal-free con A (as well as to native con A) prevents the thermal aggregation of the protein. In addition, supplementation of native con A with Mn^{2+} results in increased resistance to the effects of protein denaturants. It is known that native con A is deficient in metals[18,19]. We consider that the absence of transition metal from native con A preparations may explain the tendency for the protein to be readily denatured[5,6]. Based on the X-ray crystallographic work of Hardman[7], it is not difficult to explain why the lack of metal makes the protein marginally stable: in the absence of metal, a portion of the polypeptide strand (residues 10–20) is forced into a solvated state, because of a repulsion by side-chain carboxyl groups that bind the metal[7]. Binding of transition metal effectively neutralizes the carboxyl groups.

Our data suggest that con A is a highly stable protein when it is in the presence of an excess of a transition metal.

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