WHEAT GERM AGGLUTININ: EFFECTS OF LIMITED REDUCTION OF THE DISULFIDE BONDS AND CARBOXYMETHYLATION ON THE PROPERTIES OF THE LECTIN

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1. Introduction

Lectins are proteins mainly of plant origin which bind specifically to free sugars, or to the oligosaccharide moieties of glycoproteins and glycolipids (reviewed [1,2]). The lectin isolated from *Triticum vulgare* (WGA) consists of two protomers of 164 amino acid residues each [3–5]. It specifically binds GlcNAc or its $\beta 1 \rightarrow 4$-linked di- and trisaccharides [3] as well as sialic acid [6,7] and contains 4 binding sites for these ligands [5–9]. Each protomer consists of 4 structural domains containing 41 amino acid residues, among them 8 cysteines forming 4 intradomain disulfide bridges [3,8,10]. In connection with the application of heterobifunctional crosslinking reagents to lectins [11,12], the possibility of limited reduction of disulfide bonds in WGA was investigated. The lectin was treated with different amounts of DTT followed by carboxymethylation with IAA or iodoacetamide. We found that it is not possible to obtain a homogeneous preparation of partially reduced and carboxymethylated WGA. Rather, two populations of WGA are formed, one unmodified (or only slightly modified) and fully active, the other carboxymethylated almost to completion with no sugar binding capacity or hemagglutinating activity. We conclude that reduction of WGA is an 'all-or-none' process, in that once reduction of a molecule starts it continues to completion.

Abbreviations: WGA, wheat germ agglutinin; app. M_r, apparent relative molecular mass; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_2$, *N,N'*-di-acetylchitobiose; $(GlcNAc)_3$, *N,N',N''*-tri-acetylchitotriose; DTT, dithiothreitol; IAA, iodoacetic acid; $MeUmb(GlcNAc)_2$, 4-methylumbelliferyl-*N,N'*-diacetyl-β-chitobioside; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline (0.156 M NaCl, 10 mM phosphate, pH 7.2)

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2. Materials and methods

WGA was purchased from Makor Chemicals, Jerusalem and used without further purification. Iodo-[2-^{14}C]acetic acid (Amersham Radiochemicals) was diluted with sodium iodoacetate to spec. act. 0.1 mCi/mmol.

^{14}C Radioactivity was determined in Triton X-100/toluene/PPO/POPOP in a Packard Tricarb liquid scintillation counter. Amino acid analysis of WGA preparations, hydrolyzed in 6 N HCl at 110°C for 24 h in vacuo, was performed on a Dionex D502 amino acid analyzer.

For reduction and carboxymethylation, WGA was dissolved in 0.5–5 ml Tris–HCl buffer (50 mM (pH 7.6) sometimes containing carbohydrates as indicated) to 2.0–2.5 mg/ml final conc. DTT in a small volume of buffer was added in a 5–500-fold molar excess over WGA, followed after 4 h by a 10–1000-fold molar excess of IAA (2 mol IAA/mol DTT) in the Tris buffer. After 8 h at room temperature in the dark, the reaction mixture was dialyzed at 4°C against several changes of PBS. The extent of carboxymethylation was calculated from the amount of I[^{14}C]AA incorporated into a measured quantity of protein, (determined [13] with bovine serum albumin as standard), or by amino acid analysis from the molar ratio of S-carboxymethylcysteine and glutamic acid.

Carboxymethylated WGA was fractionated by affinity chromatography [14]. The hemagglutinating activity of WGA was determined by a serial dilution

assay in microtiter plates [15] using a 1% suspension of washed, human AB Rh^+ red blood cells. The lowest concentration of native WGA that gave complete hemagglutination under these conditions was 15–30 μg/ml.

Quantitative precipitation of WGA with polylysine–$(GlcNAc)_2$ conjugates was according to [16].

The binding parameters for native and carboxymethylated WGA were determined from Scatchard plots obtained by measuring the quenching of the fluorescence of 4 μM and 10 μM $MeUmb(GlcNAc)_2$ in continuous titrations [9], using an Aminco SPF-500 spectrofluorimeter in ratio mode. Mixtures of WGA and ligands at higher concentrations gave turbid solutions. Fluorescence was excited in the 320–336 nm range and emission was measured at 373 nm. Absorption in the 0.5 X 0.5 X 4.5 cm cuvettes used was <0.025.

Polyacrylamide gel electrophoresis was performed in the presence of 2% SDS and 5% β-mercaptoethanol with 12.5% acrylamide in the separating gel [17]. The gels were stained with Coomassie blue.

WGA concentrations were determined at 280 nm using the extinction coefficient 1.27 cm^2/mg [18], and are expressed on the basis of M_r 36 000. Modification had no noticeable influence on the shape of the protein absorption spectrum. All solutions for titrations were made up in 0.1 M sodium acetate buffer (pH 4.7) containing 0.5 M NaCl.

3. Results

The extent of carboxymethylation of WGA with IAA depends on the concentration of DTT (fig.1). Reduction with a 300–500-fold molar excess of DTT over WGA and subsequent carboxymethylation results in complete modification even in the absence of denaturing agents like urea or SDS. However, addition of 0.2 M *N*-acetyl-D-glucosamine has a strong protective effect; it diminishes the extent of modification by ~50% over the concentration range of DTT and IAA tested (fig.1). Protection against modification is obtained also with 10 mM GlcNAc. No such effect is observed with D-mannose, a sugar which does not bind to WGA. This indicates that specific blocking of the sugar binding site or even a part of it [3,5] protects some of the disulfide bridges against reduction by DTT.

Electrophoresis in polyacrylamide gels in the pres-

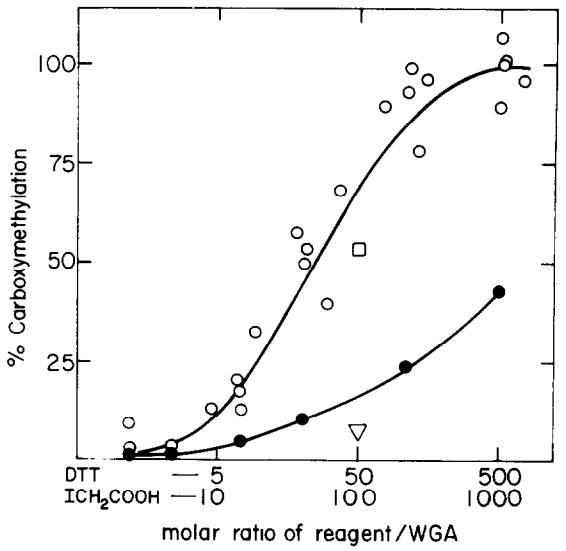

Fig.1. Reduction and carboxymethylation of WGA in the absence of sugar (○), in the presence of 0.2 M GlcNAc (●), 0.01 M $(GlcNAc)_3$ (v), and 0.2 M D-mannose (□). WGA (0.5 mg) in Tris buffer (0.2 ml, 50 mM, pH 7.6) was incubated at room temperature with DTT present in the indicated ratio. After 4 h, two equivalents of I[^{14}C]AA (100 μCi/mmol)/DTT equivalent were added in 40 μl and the incubation continued for 8 h in the dark. The samples were dialysed and the extent of carboxymethylation was calculated as described in text.

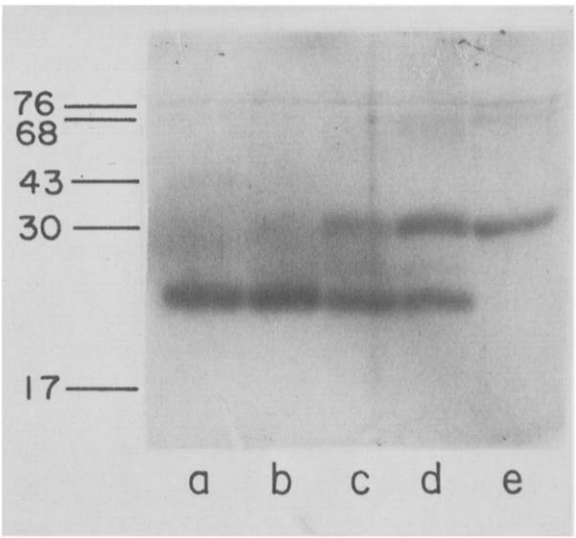

Fig.2. Polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol of WGA (a) and reduced and carboxymethylated WGA (DTT/WGA molar ratio: 5 (b); 10 (c); 20 (d); 100 (e)). M_r standards: transferrin 76 000; bovine serum albumin 68 000; ovalbumin 43 000; soybean agglutinin 30 000; myoglobin 17 000.

ence of SDS and β-mercaptoethanol shows a single band of app. M_r 22 000 [19,20] for unmodified WGA (fig.2). After partial carboxymethylation a second band with app. M_r 30 000 is observed. Its intensity relative to the first one increases with the degree of carboxymethylation, and upon complete carboxymethylation only the 30 000 M_r band is seen (fig.2). No products of intermediate electrophoretic mobility are detected. This indicates that limited reduction and carboxymethylation results in formation of two different protein species, one apparently unmodified, the other modified. The decrease of electrophoretic mobility can be related to the covalent binding of negatively-charged groups to the protein, as has also been observed with maleylated proteins [21]. Indeed, when DTT-reduced WGA is treated with the neutral iodoacetamide instead of IAA, no change in the electrophoretic mobility occurs, while the extent of alkylation and the resulting loss of sugar binding activity are the same as with IAA (not shown).

An increasing degree of modification of WGA is accompanied by a gradual decrease of activity as ascertained by assays of hemagglutination, precipitation of polylysine–$(GlcNAc)_2$ and titration of $MeUmb(GlcNAc)_2$ fluorescence (fig.3).

Also, the corresponding linear Scatchard plots of the binding to $MeUmb(GlcNAc)_2$ (not shown) demonstrates a gradual decrease of the apparent number of binding sites.

When WGA is reduced with a 5- or 35-fold molar excess of DTT in the absence of an inhibitor or with a 500-fold excess of DTT in the presence of 0.2 M *N*-acetyl-D-glucosamine and then carboxymethylated, each of the preparations obtained is separable by affinity chromatography into two fractions. The affinity adsorbed fraction obtained from the lectin modified in the absence of the inhibitory sugar, binds $MeUmb(GlcNAc)_2$ with the same association constant as native WGA (fig.4) and does not contain any *S*-carboxymethylcysteine (table 1). However, when the reduction is done in the presence of the protecting sugar, one to two disulfide bonds are reduced and alkylated without loss of sugar binding activity. Table 1 further shows that while the amount of WGA which is not adsorbed increases with the DTT/WGA ratio, the extent of carboxymethylation in this fraction is invariably high, and reaches >90% when a large excess of reagents is used. No other amino acid besides cysteine is modified to any significant extent under the conditions of reduction and alkylation used

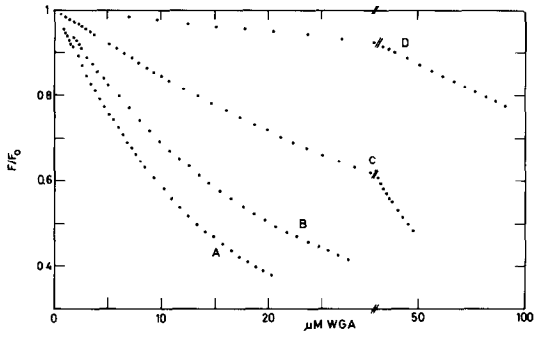

Fig.3. Dilution-corrected titration curves of total MeUmb-$(GlcNAc)_2$-fluorescence quenching. The data are expressed as relative fluorescence (F/F_0) and were obtained at pH 4.7 and 16.3°C by continuous addition of solutions (≤125 μl) of the different WGA samples from a microburette into 0.627 ml 10.0 μM $MeUmb(GlcNAc)_2$ contained in a stirred 0.5 × 0.5 × 4.5 cm fluorescence cuvette: native WGA (A); reduced and carboxymethylated WGA with DTT/WGA ratios of 5 (B), 35 (C) and 500 (D).

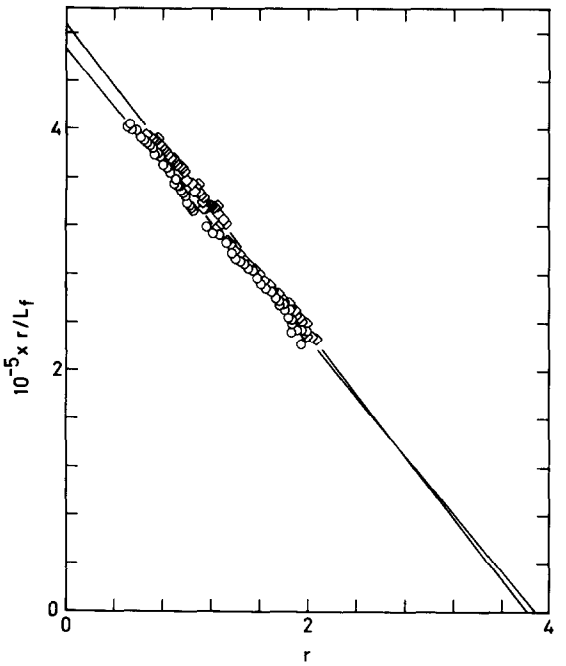

Fig.4. Scatchard plots for binding of $MeUmb(GlcNAc)_2$ to native WGA (○) and to the affinity-adsorbed fraction of WGA that was reduced and carboxymethylated (DTT/WGA = 35) (◇). The data were obtained by fluorescence titration of 4 μM and 10 μM $MeUmb(GlcNAc)_2$ as in fig.3. For both samples of WGA they correspond to 3.8 binding sites/36 000 M_r with an association constant of 1.18×10^5 M^{-1}. *r* is the ratio of bound ligand to total protein, and L_f is the concentration of free $MeUmb(GlcNAc)_2$ at equilibrium [9].

Table 1
Amino acid analysis of carboxymethylated WGA after separation by affinity chromatography[a]

	DTT/WGA = 5		DTT/WGA = 35		DTT/WGA = 500 (+0.2 M GlcNAc)	
	I[b]	II	I	II	I	II[c]
% Protein in fraction	5	95	45	55	30	70
Carboxymethyl-cysteine	12	0	28	0	20	3
Asp	15	18	16	17	16	17
Thr	5	4	5	4	5	5
Ser	17	14	14	14	14	14
Glu	19	19	19	19	19	19
Pro	12	8	7	6	6	6
Gly	41	43	43	41	43	44
Ala	10	10	10	10	10	10
Cys	2	14	0	5	4	10
Val	1	1	1	1	1	1
Met	3	2	2	2	1	1
Ile	2	2	2	2	2	2
Leu	4	4	4	4	4	4
Tyr	5	7	7	5	7	6
Phe	3	3	3	3	3	3
His	2	2	2	2	2	2
Lys	7	8	7	7	8	8
Arg	5	5	5	5	5	5

[a] WGA (5 mg) was reduced and carboxymethylated as described. After dialysis against 0.15 M NaCl it was applied to a column of Sepharose–2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine (1.5 × 7 cm) [14]. Unadsorbed WGA was eluted with 0.15 M NaCl (35 ml (I)). The adsorbed WGA was eluted with 0.1 M acetic acid (II). The amino acid composition was calculated relative to glutamic acid (19 residues/molecule)

[b] We have no ready explanation for the high amounts of serine and proline detected in this sample. However, as this fraction comprises only 5% of the total protein added to the column, analysis had to be done on a very small amount of material and even small amounts of contaminating proteins could have a great influence on the amino acid composition of the fraction

[c] This fraction consists of two protein species (see fig.5A(b,e))

(table 1). Furthermore, the incorporation of [^{14}C]-acetate into unreduced WGA is negligible.

After reduction in the absence of sugar, the protein which does not bind to the column appears as a single band of app. M_r 30 000 (fig.5A(c)) and the adsorbed WGA at M_r 22 000 (fig.5A(b)). These findings strongly suggest that reduction of a WGA molecule is an 'all-or-none' process. In the presence of SDS, reduction appears to be random since a smear of stained material can be seen after gel electrophoresis (fig.5B(d)). Under these conditions, the average app. M_r of the diffuse band shifts from 22 000 to 30 000 with increase in the DTT/WGA ratio. When native WGA is reduced in the presence of *N*-acetyl-D-glucosamine, three bands with app. M_r of 30 000, 25 000 and 22 000 can be discerned (fig.5A(d)). The new band of M_r 25 000 appears already at low DTT/WGA ratios, while the band at M_r 30 000 appears only at high DTT concentrations (compare fig.5A(d) with fig.5B(b)). The intermediate band binds strongly to the affinity column and only a small amount is washed off at neutral pH (compare fig.5A(e) and (f)). It should be noted that when β-mercaptoethanol is omitted from the gel electrophoresis buffer, native WGA and the partially reduced and carboxymethylated WGA cannot be detected by Coomassie blue

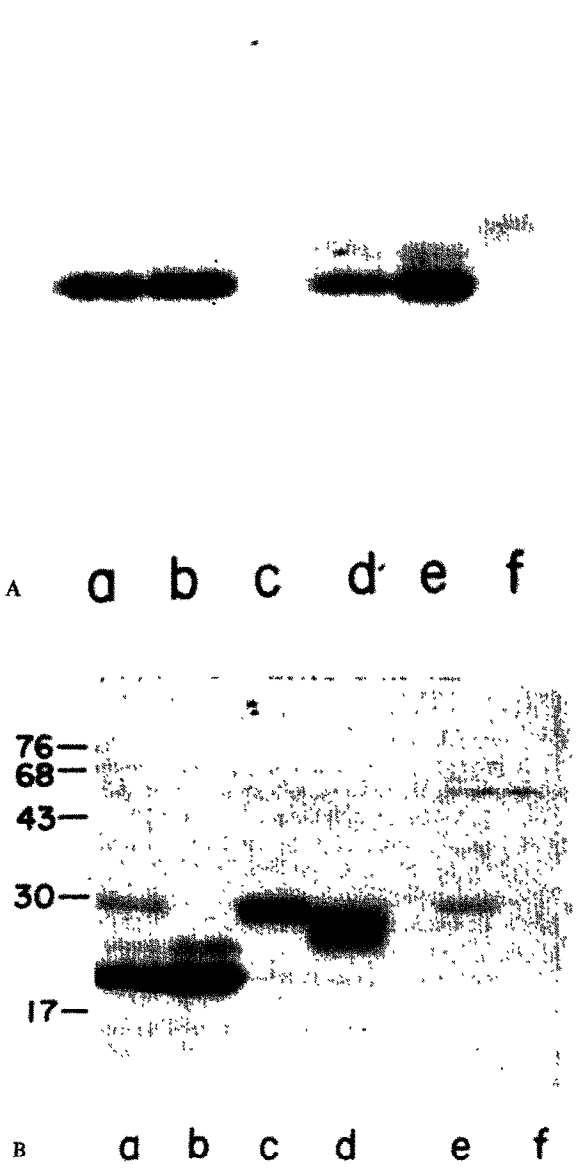

Fig.5. (A) Polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol of reduced and carboxymethylated WGA before (a,d) and after (b,c,e,f) separation by affinity chromatography. Reduction was done in the absence of sugar at DTT/WGA = 35 (a–c) and in the presence of 0.2 M GlcNAc at DTT/WGA = 500 (d–f). Specifically bound fractions (b,e), and unbound fractions (c,f). For details see table 1. (B) Polyacrylamide gel electrophoresis of reduced and carboxymethylated WGA. Samples (a–d) were heated for 3 min at 90°C in the presence of 4% β-mercaptoethanol prior to electrophoresis. Samples (e,f) were not heated and β-mercaptoethanol was omitted. (a,e) Reduction without sugar at DTT/WGA = 50; (b,f) reduction in the presence of 0.2 M GlcNAc, DTT/WGA = 50; (c,d) reduction in presence of 2% SDS, DTT/WGA = 125 (c), DTT/WGA = 32 (d).

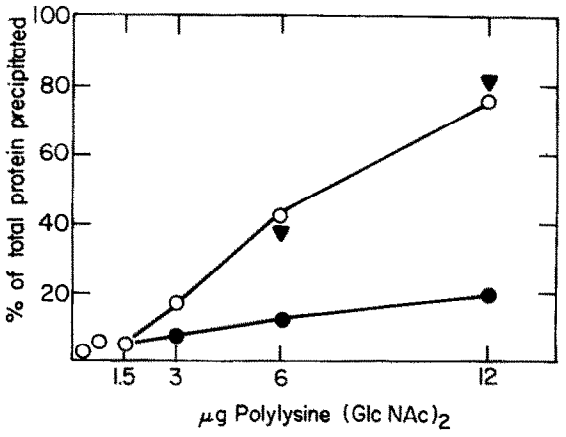

Fig.6. Precipitation with polylysine–$(GlcNAc)_2$ (0.24 mg/ml) of carboxymethylated WGA which was separated by affinity chromatography into bound (•) and unbound (○) fractions. Control precipitation with native WGA (▾). Assay was according to [17], with 20 μg lectin/tube, final vol. 500 μl.

staining, either because they do not stain or, more likely, because they do not bind SDS and consequently do not move into the gel.

The ability of the unadsorbed WGA to form a precipitate with polylysine-$(GlcNAc)_2$ is diminshed ⩾4-fold compared with that of the adsorbed material and native WGA (fig.6). The unadsorbed, carboxymethylated WGA is not hemagglutinating at ⩽0.5 mg/ml, nor does it inhibit hemagglutination when added in a 4-fold excess to native WGA. This indicates that the lack of hemagglutinating activity upon cleavage of the disulfide bonds is due to the destruction of the sugar binding sites. Finally, the Scatchard plot for binding of $MeUmb(GlcNAc)_2$ to native and to absorbed WGA cannot distinguish between the two proteins in terms of the number of binding sites and the association constant (fig.4). This clearly demonstrates that in the reduced and carboxymethylated WGA the absorbed fraction is fully active. The apparent decrease of binding sites upon titration of modified and unseparated WGA results from a mixture of active and inactive species.

4. Discussion

WGA is an unusual protein because of its very high content of cysteine residues (20 mol%), all forming intrachain disulfide bonds. Only a few other proteins are known to contain a comparable amount of cysteine. Some have alignments of S–S bridges similar

to WGA, e.g., hevein, ragweed pollen allergen and erabutoxin, a snake venom protein with structure similar to WGA [10,22]. An important function of disulfide bonds is the maintenance of protein structure; even partial reduction of such bonds in a protein usually results in decreased stability against denaturation and proteolysis, and in loss of biological activity [23]. Disulfide bonds in proteins can be quite resistant against reduction, and complete reduction sometimes requires the presence of denaturing agents. In partially reduced proteins it is not always easy to distinguish if loss of reactivity is due to partial modification of all the molecules to the same extent, or to formation of a mixture of completely reduced and inactive and unreduced, active molecules. The latter possibility applies to reduction and carboxymethylation of S–S bridges in WGA.

We have shown that WGA can be completely reduced in the absence of denaturing agents, and that specific binding of sugars to the lectin protects against reduction. Moreover, the partially reduced and carboxymethylated product proved to be a mixture of essentially two species, the unmodified protein and fully reduced and carboxymethylated WGA, suggesting that the reduction in this case is an 'all-or-none' process, i.e., once the first S–S bridge is opened, the remaining ones are reduced very easily.

Little is known about the biosynthesis and physiological function of lectins in plants [2]. It is possible that the disulfide bonds are formed on the nascent polypeptide chain, each time the translation of a domain is completed. This would agree with the observation of spontaneous refolding and reoxidation of reduced erabutoxin [24] which has a folding pattern equivalent to that of one of the 4 homologous domains of WGA [7,10].

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