Studies on chemical modification of cold agglutinin from the snail *Achatina fulica*

Manju SARKAR, Debashis MITRA and Asish K. SEN

Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Calcutta-700032, India

The cold agglutinin isolated from the albumin gland of the snail *Achatina fulica* was modified with various chemical reagents in order to detect the amino acids and/or carbohydrate residues present in its carbohydrate-binding sites. Treatment with reagents considered specific for modification of lysine, arginine and tryptophan residues of the cold agglutinin did not affect the carbohydrate-binding activity of the agglutinin. Modification of tyrosine residues showed some change. However, modification with carbodiimide followed by α -aminobutyric acid methyl ester causes almost complete loss of its binding activity, indicating the involvement of aspartic acid and glutamic acid in its carbohydrate-binding activity. The carbohydrate residues of the cold agglutinin were removed by β -elimination reaction, indicating that the sugars are *O*-glycosidically linked to protein part of the molecule. Removal of galactose residues from the cold agglutinin by the action of β -galactosidase indicated that the galactose molecules are β -linked. These carbohydrate-modified glycoproteins showed a marked change in agglutination property, i.e. they agglutinated rabbit erythrocytes at both 10 °C and 25 °C, indicating that the galactose residues of the glycoprotein play an important role in the cold-agglutination property of the glycoprotein. The c.d. data showed the presence of an almost identical type of random-coil conformation in the native cold agglutinin at 10 °C and in the carbohydrate-modified glycoprotein at 10 °C and 25 °C. This particular random-coil conformation is essential for carbohydrate-binding property of the agglutinin.

INTRODUCTION

Cold agglutinins, predominantly human IgM antibodies, are obtained largely from patients with cold-agglutinin disease. Some of these agglutinins showed specificity for linear (i-antigen) or branched (I-antigen) chains of poly-lactosamine (Hakomori, 1981). The cold agglutinin from the albumin gland of snail *Achatina fulica* was purified and characterized as a glycoprotein (M_r 220 000) consisting of three non-covalently bound subunits (M_r 84 000, 74 000 and 62 000). The predominant amino acids were aspartic acid, glutamic acid and serine, and D-galactose was the major sugar component. *N*-Acetylglucosamine, *N*-acetylgalactosamine and small amounts of mannose, xylose and fucose were also present. The cold agglutinin has the property of binding β -galactoside, and *N*-acetyl-lactosamine was the most potent inhibitor (Sarkar *et al.*, 1984; Mitra *et al.*, 1987).

In the present study the chemical nature of the binding site of the glycoprotein was explored by modifying the amino acids and the carbohydrate residues with various reagents. The nature of the bond involved between carbohydrate and protein part of the glycoprotein was also investigated by chemical method. Finally some light is thrown on the cold-agglutination property of the agglutinin.

MATERIALS AND METHODS

All the chemicals were of analytical grade or of the purest material available. Cyclohexane-1,2-dione, 2-hydroxy-5-nitrobenzyl bromide, α -aminobutyric acid methyl ester hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride, α -galactosidase (EC 3.2.1.22) from *Aspergillus niger* and

β -galactosidase (EC 3.2.1.23) from *Escherichia coli* were purchased from Sigma Chemical Co. The agglutinin was isolated and purified from the albumin gland of *Achatina fulica* snail by affinity chromatography on sheep gastric mucin-Sepharose 4B as described previously (Mitra *et al.*, 1987). The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951), with crystalline bovine serum albumin (globulin-free, lot 61F-9335; Sigma) as standard or by measuring the absorbance at 280 nm [an $A_{1\%}^{1\text{cm}}$ value of 13.4 was assumed (Mitra *et al.*, 1987)].

Haemagglutination was performed both at 10 °C and 25 °C with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, VA, U.S.A.) using 25 μ l loops and 25 μ l of a 2% rabbit erythrocyte suspension. The end point was determined visually 1 h after the addition of erythrocytes. The reciprocal dilution of this end-point concentration of protein which produces visible agglutination gives the titre.

Carbohydrate analysis

G.l.c. was performed with a Hewlett–Packard 5730A gas chromatograph fitted with a flame-ionization detector. Resolutions were performed on glass columns (2 m $\times$ 6 mm) containing (a) 3% of ECNSS–Mono Gas Chrom Q (100–200 mesh) at 190 °C or (b) 3% of Poly A-103 on Gas Chrom Q (100–200 mesh) at 200 °C. Paper chromatography was performed on Whatman no. 1 paper, with the following solvent systems: (A) ethyl acetate/pyridine/water, (8:2:1, by vol.) and (B) butan-1-ol/pyridine/water (6:4:3, by vol.). The sugars were detected with alkaline silver nitrate reagent (Sen *et al.*, 1986). For neutral-sugar analysis, 4 mg of sample was hydrolysed with 2 M-trifluoroacetic acid for 2 h at 120 °C in a sealed tube (Albersheim *et al.*, 1967). After

hydrolysis, D-allose was added as internal standard and trifluoroacetic acid was removed under vacuum. Traces of acid were removed by co-distillation with water. The hydrolysate was then reduced with NaBH_4 and converted into alditol acetate (Sloneker, 1972). The neutral-sugar alditol acetates were detected and estimated by g.l.c. on column (a) described above.

For the detection of amino sugars, 4 mg of sample was hydrolysed with 4 M-HCl for 6 h at 100 °C (Conchie, 1976). Allose, as internal standard, was added to the hydrolysate and the acid was removed under vacuum. Last traces of acid was removed by co-distillation with water. The hydrolysate was then reduced with NaBH_4 , neutralized with acetic acid and converted into alditol acetate and analysed on column (b) at 200 °C.

Immunodiffusion

The antiserum to crude extract was prepared as reported previously (Mitra *et al.*, 1987). Immunodiffusion was carried out by using purified and/or modified agglutinin and antiserum as described by Ouchterlony (1948).

Binding to sheep gastric mucin-Sepharose 4B column

Cold agglutinin or its modified derivatives was loaded individually on a sheep gastric mucin-Sepharose 4B column (5 cm $\times$ 1 cm) equilibrated with phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.2, containing 150 mM-NaCl) and washed with the buffer at 10 °C. The bound protein was eluted by washing the column with the same buffer at 25 °C.

C.d. analysis

The c.d. spectra of purified cold agglutinin (0.1 mg/ml) and its modified derivatives (0.11 mg/ml) were measured in a cell of 1 ml capacity and 2 mm path length in the wavelength region 300–190 nm with a Jasco J-20A recording spectropolarimeter. These data are expressed in terms of mean residue ellipticities $[\theta]$ in degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ as described previously (Mitra *et al.*, 1987). All recordings were performed in phosphate-buffered saline. The errors due to noise were ± 2 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

Chemical modification of cold agglutinin

All the treatments were performed under mild conditions and with reagents that could modify particular amino acids. The bulk of the purified agglutinin was dialysed against 0.9% NaCl to remove phosphate ions and was used for chemical modifications.

(a) Acetylation. A 3 ml portion of the agglutinin (2 mg) was diluted to 6 ml with 50% -satd. sodium acetate, pH 8.3, at 0 °C. Acetic anhydride was then added in four 1 μl portions over a period of 1 h, the pH being maintained at 8.3 by the addition of 0.1 M-NaOH. It was then dialysed first against distilled water and finally against phosphate-buffered saline at 4 °C (Rice & Etzler, 1975).

(b) Citraconylation. A 3 ml portion of the agglutinin (2 mg) was diluted to 6 ml with 0.1 M-Tris/HCl buffer, pH 8.0, at 20 °C. Citraconic anhydride (9 μl) was added in three portions with constant stirring at 20 °C over a period of 30 min. The pH was maintained between 8 and 9 by the addition of 0.1 M-NaOH. After 1 h the solution

was dialysed exhaustively against 5 mM-Tris/HCl buffer, pH 8.6, at 4 °C (Dixon & Perham, 1968).

(c) Treatment with 2-hydroxy-5-nitrobenzyl bromide. A 3 ml portion of the agglutinin (2 mg) was diluted with 3 ml of 0.28 M-acetic acid, pH 2.7. A solution (100 μl) of 2-hydroxy-5-nitrobenzyl bromide in dry acetone (50 mg/ml) was added in five equal portions to the above solution for a period of 1 h. The pH was maintained below 3 by the addition of acetic acid. The solution was passed through a column of Sephadex G-25 (10 cm $\times$ 1 cm) equilibrated with 0.15 M-acetic acid. The fractions containing the protein were pooled and dialysed exhaustively against distilled water and finally against phosphate-buffered saline at 4 °C (Horton & Koshland, 1972).

(d) Modification with cyclohexane-1,2-dione. The agglutinin (3 ml), containing 2 mg of protein, was diluted to 6 ml by adding 0.4 M-sodium borate buffer, pH 9.0, containing 0.05 M-cyclohexane-1,2-dione. The reaction mixture was flushed with N_2 and then incubated at 37 °C in the dark for 4 h. The reaction was terminated by the addition of 1 ml of 5% (v/v) acetic acid in water (Patthy & Smith, 1975). The reaction mixture was dialysed against water and finally against phosphate-buffered saline.

(e) Carbodi-imide reaction. A 3 ml portion of the agglutinin (2 mg) was diluted to 6 ml with 1 M- α -aminobutyric acid methyl ester, pH 4.7. Two 50 μl portions of 1.1 M-1-ethyl-3-(3-dimethyl-aminopropyl)-carbodi-imide were added at 30 min intervals. The mixture was stirred constantly and the pH was maintained at 4.7 by the addition of HCl. After 5.5 h, the reaction mixture was dialysed exhaustively against distilled water and finally against phosphate-buffered saline at 4 °C (Carraway & Koshland, 1972).

(f) Modification of carbohydrate group by β -elimination reaction. A 3 ml portion of the agglutinin (2 mg) was diluted with 3 ml of 0.1 M-NaOH and then 0.05 M- NaBH_4 (3 ml) was added at 25 °C. After 30 h it was acidified with acetic acid and dialysed against distilled water (Iyer & Carlson, 1971) and finally against phosphate-buffered saline. The material outside the dialysis bag and the modified agglutinin were analysed for neutral and amino sugars by g.l.c. as described above.

(g) Treatment with α -galactosidase. A 3 ml portion of the agglutinin (2 mg) was diluted to 6 ml with 0.1 M-phosphate buffer, pH 6.0, and incubated with 100 μl (2 units) of α -galactosidases at 37 °C for 7 days in presence of toluene. It was then dialysed against phosphate-buffered saline (Allen *et al.*, 1978) at 10 °C and the dialysis diffusate was collected and concentrated. Sugar analysis of the material was then performed by paper chromatography.

(h) Treatment with β -galactosidase. A 3 ml portion of the agglutinin (2 mg) was diluted to 6 ml with phosphate-buffered saline and incubated with 4 μl (16 units) of enzyme at 37 °C for 24 h in the presence of toluene and finally dialysed against phosphate-buffered saline at 10 °C (Allen *et al.*, 1978). The dialysis diffusate was collected, concentrated to a small volume and analysed for sugars by paper chromatography.

Table 1. Effect of chemical modification on haemagglutinating and carbohydrate-binding properties

'Specific activity' is defined as the total haemagglutination titre/mg of protein. The reciprocal of the highest dilution of the agglutinin that produces visible agglutination was taken as the titre. The binding to sheep gastric mucin-Sepharose 4B (SGM-Seph.) was tested by passing the protein through the column (5 cm × 1 cm) and eluting the protein. '+' indicates binding to the affinity column; SB, slightly bound; NB, not bound.

Modification	Type of group modified	Specific activity (Titre/mg of protein)		Binding to the SGM-Seph. column
		10 °C	25 °C	
Native		4977	0	+
Acetic anhydride	Lys ϵ -amino, <i>N</i> -terminal	2087	0	SB
	α -amino and Tyr hydroxy			
Citraconic anhydride	Lys ϵ -amino and <i>N</i> -terminal	4977	0	+
	α -amino			
2-Hydroxy-5-nitro-benzyl bromide	Trp indole ring	4977	0	+
Carbodi-imide, then α -aminobutyric acid methyl ester	Glu γ -carboxy, Asp β -carboxy and <i>C</i> -terminal α -carboxy	33	0	NB
Cyclohexane-1,2-dione	Arg guanido	4977	0	+
NaOH/NaBH ₄	Removal of carbohydrate	35400	4977	+
α -Galactosidase	Removal of α -galactose	4977	0	+
β -Galactosidase	Removal of β -galactose	35400	4977	+

Both the enzyme-treated samples were passed through a sheep gastric mucin-Sepharose 4B column at 10 °C to remove the enzymes. The bound agglutinins were eluted from the column at 30 °C with phosphate-buffered saline or with 100 mM-galactose in phosphate-buffered saline at 10 °C.

RESULTS

The specific activity and the carbohydrate-binding properties of the native cold agglutinin and its modified derivatives are summarized in Table 1. Haemagglutinations were checked both at 10 °C and 25 °C. Modification, in cold agglutinin, of the ϵ -amino group of lysine, of the *N*-terminal α -amino group and of the hydroxy group of tyrosine with acetic anhydride produced a decrease in specific activity, whereas treatment with citraconic anhydride, which modifies only the ϵ -amino group of lysine and the *N*-terminal amino group, had no effect. This suggests that the tyrosine residue makes some contribution to the binding activity of the agglutinin. The specific activity remained more or less unchanged when the cold agglutinin was treated with 2-hydroxy-5-nitrobenzyl bromide, a reagent that modifies tryptophan residues, and with cyclohexane-1,2-dione, which modifies the guanido group of arginine. Modification of amino acid residues of the cold agglutinin does not produce any change in the specific activity of the agglutinin at 25 °C, i.e. the cold agglutinin, as well as the modified agglutinins so far discussed, do not agglutinate erythrocytes at 25 °C. The agglutinin is highly sensitive to 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide followed by α -aminobutyric acid methyl ester, and it loses almost all its specific activity, indicating the presence of glutamic acid, aspartic acid and the *C*-terminus in the carbohydrate-binding site.

All these results were further confirmed by passing all the modified agglutinins through the sheep gastric

mucin-Sepharose 4B column. All the modified forms of cold agglutinin, excluding the carbodi-imide-modified form, behaved as the cold agglutinin, i.e. they bound to the affinity column at 4 °C and were eluted at 25 °C. The carbodi-imide-modified cold agglutinin failed to bind with the affinity matrix.

The carbohydrate groups of the purified cold agglutinin were removed by a β -elimination reaction (Iyer & Carlson, 1971). Carbohydrate analysis of modified cold agglutinin by g.l.c. showed that all the carbohydrate residues were removed by the β -elimination reaction. From Table 1 it is evident that the carbohydrate-modified agglutinin agglutinates both at 10 °C and 25 °C, although the specific activity of the agglutinin is about seven times higher at 10 °C than at 25 °C and it binds to the sheep gastric mucin-Sepharose 4B column. The modified agglutinin was eluted with 100 mM-galactose at 10 °C or with phosphate-buffered saline at 25 °C. The cold agglutinin was treated separately with α - and β -galactosidase. The excess enzymes were removed by affinity chromatography on a sheep gastric mucin-Sepharose 4B column instead of inactivating at 100 °C, which may cause deactivation of the agglutinin. Treatment of the cold agglutinin with β -galactosidase liberated free galactose, as revealed by paper chromatography of the dialysis residue, indicating that the galactose residues are β -linked. Treatment of the cold agglutinin with α -galactosidase, however, failed to liberate galactose, indicating the absence of α -glycosidic linkage. The agglutination property of the β -galactosidase-treated cold agglutinin was similar to that of the cold agglutinin modified by the β -elimination reaction, whereas α -galactosidase-treated material showed no change in agglutination properties (Table 1).

Fig. 1 shows the immunodiffusion pattern of cold agglutinin and various modified cold agglutinins against the antisera raised in rabbits by injecting crude extract of the albumin gland. The perfect fusion of precipitin lines

was observed with all modified forms, excluding carbodi-imide-modified agglutinin, which showed a somewhat different pattern on the Ouchterlony plate.

The c.d. spectra of the purified cold agglutinin and carbohydrate-modified agglutinin are shown in Fig. 2. In both cases a negative band in the far u.v. was observed. The c.d. spectrum of purified cold agglutinin (0.1 mg/ml) exhibits two troughs, at 208 nm and 204 nm, with the

Fig. 1. Immunodiffusion pattern of the purified agglutinin and its modified derivatives

Well 1, rabbit antiserum to crude albumin-gland extract; well 2, purified cold agglutinin; well 3, carbohydrate-modified agglutinin (NaOH/NaBH₄ treatment); well 4, tryptophan-modified agglutinin (2-hydroxy-5-nitrobenzyl bromide treatment); well 5, arginine-modified agglutinin (cyclohexane-1,2-dione treatment); well 6, amino-group-modified agglutinin (citraconic anhydride treatment); well 7, carboxy-group-modified agglutinin (carbodi-imide treatment).

Fig. 2. C.d. spectra of cold agglutinin and its carbohydrate-modified derivative at 25° and 10 °C

C.d. spectrum of cold agglutinin: a, at 25 °C; b, at 10 °C; c, spectrum of carbohydrate-modified agglutinin at 25 °C; d, at 10 °C.

ellipticity values -4300 and -5400 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ respectively (Fig. 2, spectrum a) at 25 °C, and only one trough at 205 nm, with an increased ellipticity value of -10000 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ at 10 °C (Fig. 2, spectrum b). The c.d. spectrum of carbohydrate-modified cold agglutinin (0.1 mg/ml) exhibits troughs at 203 nm of ellipticity values -7400 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ at 25 °C (Fig. 2, spectrum c) and -8800 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ at 10 °C (Fig. 2, spectrum d). This result shows that at 10 °C and 25 °C there is no significant change in the conformation of the modified agglutinin and that the pattern is similar to that of the purified cold agglutinin at 10 °C.

DISCUSSION

Chemical modification of the purified cold agglutinin with various amino-acid-modifying agents shows that the C-terminal groups of amino acids, the carboxy groups of glutamic acid and aspartic acid and some tyrosyl groups are present in the carbohydrate-binding site of the agglutinin. It was reported previously (Mitra *et al.*, 1987) that the cold agglutinin has a high glutamic acid and aspartic acid content. So these two amino acids are the main constituents of the carbohydrate-binding site of the agglutinin. We also reported that the cold agglutinin is a glycoprotein containing galactose (14.4%), *N*-acetylglucosamine (8.1%) and *N*-acetyl-galactosamine (8.1%). A small amount of mannose, fucose and xylose was also present. Chemical modification of the cold agglutinin by the β -elimination reaction removed the sugar moieties from the cold agglutinin (as revealed by g.l.c.). The modified agglutinin agglutinates rabbit erythrocytes both at 10 °C and 25 °C, the nature of the molecule being apparently maintained unchanged as suggested by immunodiffusion and binding to the sheep gastric mucin-Sepharose 4B column. As the β -elimination reaction removes all the carbohydrate moieties, it can be assumed that the sugars are linked to protein of the cold agglutinin *O*-glycosidically and that these carbohydrate moieties play an important role in cold-agglutinating property of the agglutinin. This was further confirmed by c.d. studies. The c.d. spectra of the carbohydrate-modified agglutinins at 10 °C and 25 °C were almost similar to that of cold agglutinin at 10 °C. This suggests that the carbohydrate-modified cold agglutinin acquires a random-coil conformation at 10 and 25 °C similar to that of the native cold agglutinin at 10 °C and that this conformation is essential for the carbohydrate-binding property. Treatment of cold agglutinin with α - and β -galactosidases showed that β -galactosidase-treated cold agglutinin behaves similarly to carbohydrate-modified agglutinin, whereas α -galactosidase-treated glycoprotein behaves like native cold agglutinin. Galactose was liberated from the cold agglutinin only by the action of β -galactosidase. This result indicates that the galactose residues are present as β -anomers in the glycoprotein. The presence of these β -galactoside residues are important for the cold-agglutination properties of the glycoprotein. The presence of β -galactose in an agglutinin having a β -galactoside-binding property is quite unusual in the literature, except for human monoclonal IgM (κ) (cold agglutinin MKV), which is a sialoglycoprotein that binds to sialic acid (Tsai *et al.*, 1977). The perfect fusion of precipitin lines on immunodiffusion suggests a

complete cross-reactivity between native cold agglutinin and all its modified forms, except carbodi-imide-modified agglutinin.

In conclusion, the present study suggests that the carbohydrate-binding sites of the cold agglutinin is in the protein part of the molecule and that glutamic acid and aspartic acid are probably present in the binding site; for cold-agglutination behaviour, however, the carbohydrate part of the molecule plays an important role.

This work was supported by the Council of Scientific and Industrial Research, India, in the form of a Senior Research Fellowship to D.M. We thank Dr. S. C. Pakrashi, Director, I.I.C.B. (Indian Institute of Chemical Biology), for his interest in the work. We thank Professor B. K. Bachhawat, Ex-Director, I.I.C.B., and present head of the Department of Biochemistry, University of Delhi, for his constant encouragement and useful suggestions during the course of the work. The helpful suggestions of Dr. M. Chaudhuri and Dr. C. Mandal are also acknowledged.

REFERENCES

- Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967) *Carbohydr. Res.* **5**, 340–345
- Allen, A. K., Desai, N. N. & Neuberger, A. (1978) *Biochem. J.* **171**, 665–674
- Carraway, C. & Koshland, D. E. (1972) *Methods Enzymol.* **25**, 618–620
- Conchie, J. (1976) *Methods Carbohydr. Chem.* **7**, 195–199
- Dixon, H. B. F. & Perham, R. N. (1968) *Biochem. J.* **109**, 312–314
- Hakomori, S. (1981) *Semin. Hematol.* **18**, 39–62
- Horton, H. R. & Koshland, D. E. (1972) *Methods Enzymol.* **25**, 468–482
- Iyer, R. N. & Carlson, D. M. (1971) *Arch. Biochem. Biophys.* **142**, 101–105
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mitra, D., Sarkar, M. & Allen, A. K. (1987) *Biochem. J.* **242**, 331–338
- Ouchterlony, O. (1948) *Acta. Pathol. Microbiol. Scand.* **25**, 186–191
- Patthy, L. & Smith, E. L. (1975) *J. Biol. Chem.* **250**, 557–564
- Rice, R. H. & Etzler, M. E. (1975) *Biochemistry* **14**, 4093–4099
- Sarkar, M., Bachhawat, B. K. & Mandal, C. (1984) *Arch. Biochem. Biophys.* **233**, 286–289
- Sen, A. K., Banerjee, N. & Farooqi, M. I. H. (1986) *Carbohydr. Res.* **157**, 251–256
- Sloneker, J. H. (1972) *Methods Carbohydr. Chem.* **6**, 20–24
- Tsai, C. M., Zopf, D. A., Yu, R. K., Wistar, R. W., Jr. & Ginsburg, V. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4591–4594

Received 29 December 1986/13 April 1987; accepted 7 May 1987