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Oxidation and chemical modification of lung β-galactoside-specific lectin

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Galaptins are small, soluble, lectins with a specificity for β-galactose residues. Many galaptins are inactivated by atmospheric oxygen and are protected by disulphide-reducing reagents. We find that each subunit of rat lung galaptin contains one residue of tryptophan and six of cysteine. Oxygen inactivates rat lung galaptin by oxidation of the cysteine residues. During oxidation, the normal dimeric structure is maintained and all disulphide bonds are formed within individual subunits. Exogenous thiols protect against inactivation, but oxidized thiols accelerate inactivation. Human lung fibroblast galaptin is almost completely inactivated within 1 h in tissue culture medium at 37 °C. Alkylation of native rat lung galaptin with iodoacetate or ethyleneimine causes substantial loss of activity. The dimeric galaptin structure is maintained. In contrast, alkylation with iodoacetamide yields carboxamidomethyl-galaptin, which is fully active and stable to atmospheric oxygen in the absence of disulphide-reducing reagents. This derivative is very useful for studies of galaptin properties and function.

In the past several years, β-galactoside-binding proteins have been described in different vertebrate species and different organs (Barondes, 1984). These proteins can be operationally defined as lectins and are usually assayed by their ability to agglutinate erythrocytes. Despite their diverse origins (Barondes, 1984), these β-galactoside-binding proteins (or 'galaptins'; Harrison & Chesterton, 1980) have many common properties including subunit M_r (13000–16000), dimeric structure, saccharide specificity and activity that is independent of bivalent cations but dependent on the presence of exogenous thiols such as 2-mercaptoethanol or dithiothreitol.

The function of these β-galactoside-binding proteins has not been elucidated. Perhaps their most obvious role might be in a cellular-adhesion process, and some results indicate that galaptins are secreted and found extracellularly (Barondes & Haywood-Reid, 1981; Beyer & Barondes, 1982; Cerra *et al.*, 1984); other results point to a predominantly intracellular location (Podleski & Greenberg, 1980; Barondes, 1981; Sanford *et al.*, 1982). An intracellular location would be more in accordance with the dependence of galaptin activity on 2-mercaptoethanol or dithiothreitol, since extracellular proteins rarely have susceptible thiol groups (Cecil, 1963).

The dependence of galaptins on exogenous thiols has been often noted but seldom studied. Levi & Teichberg (1981) reported that electrolectin, the β-galactoside-binding protein from *Electrophorus electricus*, is inactivated in the absence of exogenous thiols, even though electrolectin contains no cysteine residues. The loss of activity was traced to oxidation of the single tryptophan residue by atmospheric oxygen, a process that was prevented by addition of 2-mercaptoethanol. Those authors suggested that a similar mechanism may account for the dependence of vertebrate lectins on exogenous thiols, but they recently reported that inactivation of galaptin from chick thymus is not due to oxidation of tryptophan and can probably be attributed to a sensitive cysteine residue (Levi & Teichberg, 1985).

We have previously described the purification and some properties of human and rat lung galaptins (Powell, 1980) and demonstrated that human lung fibroblasts synthesize galaptin (Whitney *et al.*, 1985). The purified galaptins require exogenous thiols to maintain their agglutinating and oligosaccharide-binding activities. The present paper describes the quantification of tryptophan and cysteine residues and the effect of oxidation or chemical modification of the cysteine residues on the activity of lung galaptin. Of particular interest is the carboxamidomethyl(Cam)-galaptin, a modified galaptin that does not require exogenous thiols for activity.

EXPERIMENTAL PROCEDURES

Materials

Aquasol II and iodo[^{14}C]acetic acid were from New England Nuclear. [^{3}H]Phenylalanine was from ICN Chemical and Radioisotope Division; bovine serum albumin (Fraction V) and trypsin (Type II, crude) were from Sigma. Iodoacetamide was purified by precipitation from acetone with light petroleum (b.p. 35–60 °C) and dried *in vacuo*. Iodoacetic acid was titrated with NaOH and freeze-dried to yield sodium iodoacetate free of HI. Sprague–Dawley albino rats were from Charles River

Abbreviations used: Cam-, carboxyamidomethyl-; Cm-, carboxymethyl-.

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Breeding Laboratories. Frozen rat lungs were purchased from Pel-Freez Biologicals, Rogers, AR, U.S.A. Human lung fibroblasts (CCD-llLu) were purchased from American Type Culture Collection, Rockville, MD, U.S.A. and were subcultured (by Dr. W. Lichter in the Department of Microbiology of this University) in Eagle's (1959) minimal essential medium plus non-essential amino acids, Earle's (1943) salts and 10% (v/v) fetal-calf serum. Lactosyl-Sepharose CL-6B was prepared as described by Levi & Teichberg (1981). A tetrasaccharide fraction containing predominantly lacto-*N*-tetraose was prepared from human milk as described by Kobata (1972).

Methods

Rat lung galaptin was purified and assayed by haemagglutination as previously described (Whitney *et al.*, 1985). The galaptin migrated as a single band on polyacrylamide-gel electrophoresis (Laemmli, 1970) and isoelectric focusing (Wrigley, 1971).

^{3}H-labelled human fibroblast galaptin was prepared by adding 100 μCi of [^{3}H]phenylalanine (20 Ci/mmol) to each of 17 T-75 flasks of nearly confluent human lung fibroblasts growing in 10 ml of Eagle's minimal essential medium plus 10% fetal-calf serum and 5 mM-glutathione. After 15 h at 37 °C in CO_2/air (1:19), the cells were harvested, washed in phosphate-buffered saline (123 mM-NaCl/10.4 mM-Na_2HPO_4/3.2 mM-KH_2PO_4, pH 7.2) plus 1 mM-dithioerythritol, sonicated in 10 ml of 20 mM-sodium phosphate (pH 7.2)/250 mM-sucrose/10 mM-lactose/10 mM-dithioerythritol and centrifuged at 30000 *g* for 40 min. The supernatant fluid was dialysed against 20 mM-sodium phosphate buffer (pH 7.2)/140 mM-NaCl (buffer A) plus 25 mM-mercaptoethanol and purified by affinity chromatography; we used a 1.5 cm × 13 cm column of lactosyl-Sepharose, followed by dialysis and a 0.7 cm × 16 cm column of lactosyl-Sepharose (Whitney *et al.*, 1985). The galaptin had 3700 c.p.m./μg of protein.

Analytical procedures. Most samples for amino acid analysis were hydrolysed in 6M-HCl/phenol (10 mg/ml) at 110 °C for 22 h, dried, and dissolved in sodium citrate buffer, pH 2.2. To analyse for tryptophan, samples were hydrolysed in 4 M-methanesulphonic acid (Pierce Chemical Co.) for 20 h at 100 °C, titrated with 0.67 vol. of 0.56 M-sodium citrate and then analysed the usual way. Analyses were performed at 54 °C with a JEOL 5AH amino acid analyser with a 0.8 cm × 46 cm column (sulphonated polystyrene; 12±2 μm beads) and a ninhydrin detection system.

To determine the total number of cysteine residues in galaptin, the purified protein was denatured in 5.7 M-guanidinium chloride/100 mM-Tris/HCl buffer (pH 8.5)/5 mM-dithioerythritol for 1 h and then allowed to react with 20 mM-iodoacetate or -iodoacetamide for 1 h at room temperature. The solution was dialysed 3 × 2 h against buffer A plus 5 mM-dithioerythritol and 10 mM-lactose and passed through a 1.5 cm × 3.5 cm column of Bio-Gel A-0.5m run in the same buffer. The protein peak was located and portions taken for assays and for amino acid analysis; an equal volume of eluant from fractions collected before the galaptin peak was taken for amino acid analysis as a blank.

To determine the number of thiol groups, galaptin was first allowed to react for 20 min at room temperature with 33 mM-iodoacetamide in 400 mM-Tris/HCl buffer (pH 8.5)/4 mM-dithioerythritol. Solid guanidinium chloride was then added to give a final concentration of 5.6 M-guanidinium chloride and 20 mM-iodoacetamide; the reaction was allowed to proceed for a further 1 h. The sample was dialysed 3 × 2 h against buffer A plus 10 mM-lactose and passed through the Bio-Gel A-0.5m column. The protein peak was located and hydrolysed for amino acid analysis; a blank from fractions preceding the galaptin peak was also taken for amino acid analysis.

The presence of dithioerythritol complicated the use of u.v. absorption to measure low concentrations of protein, so we usually measured protein by using a modification of the dye-binding procedure of Bradford (1976). The stock reagent contained 200 mg of Coomassie Blue G, 100 ml of methanol, 200 ml of phosphoric acid and 100 ml of water; the working solution was 1 ml of stock solution per 50 ml of water. Usually 20 μl or less of sample was mixed (gently) with 800 μl of reagent and A_{595} determined soon after mixing. By using a 100 μl sample, 0.2 μg of protein could be reliably detected.

Analytical ultracentrifugation was done by Dr. J. F. Woessner and Ms. Caroline Taplin (of the Department of Biochemistry of this University), who used a Beckman Model E instrument with interference optics and the procedure described by Yphantis (1964). For use in calculation of M_r of the protein, a partial specific volume ($\bar{v}$) of 0.73 was calculated from the amino acid composition (Cohn & Edsall, 1943). Yphantis cells were used at 20 °C and at 32000 rev./min for 24 h. Comparison with photographs taken earlier showed that equilibrium had been reached. Before centrifugation the samples were dialysed for 24 h at 4 °C against buffer A plus 5 mM-dithiothreitol or, for oxidized samples, buffer A under an oxygen atmosphere. Protein concentrations ranged from 0.2 to 1 mg/ml. A portion of the oxidized sample was analysed for remaining thiol groups by reaction with iodoacetamide in concentrated guanidinium chloride as described above, except that the gel-filtration step was omitted.

Analytical slab-gel electrophoresis in the presence of SDS was done by using 11% (w/v) polyacrylamide in the separating gel as described by Laemmli (1970).

Oxidative inactivation of galaptin. The stability of human fibroblast galaptin in culture medium was studied by adding 1 μg of [^{3}H]galaptin to 4 ml of medium in a T-25 plastic tissue-culture flask. After incubation of the flask at 37 °C, usually for 1 h, galaptin activity was assessed by passing the medium through a 0.75 cm × 4 cm column of lactosyl-Sepharose, washing the column with 5 ml of buffer A plus 3 mM-dithioerythritol and then eluting with 10 mM-lactose in the same buffer. Galaptin activity was expressed as the percentage of the galaptin that bound to lactosyl-Sepharose and was eluted with lactose.

Spectral studies were done to assess the possible oxidation of the tryptophan residue in rat lung galaptin. A solution of galaptin (700 μl, 600 μg/ml) was dialysed at 4 °C against 250 ml of buffer A plus 1 mM-dithiothreitol for 2.5 h, 1000 ml of buffer A plus 0.1 mM-dithiothreitol for 2 h and then 1000 ml of buffer A with continuous flushing with N_2 for 2.5 h. After dilution to 350 μg/ml, the u.v. spectrum was determined by using a Cary 14 recording spectrophotometer. The solution was then

flushed with O_2 for 30 min at room temperature; spectra were recorded after the solution was left at room temperature for an additional 2.5 h and again after 18 h. The number of thiol groups remaining was determined by reaction with iodoacetamide in concentrated guanidinium chloride as described above under 'Analytical procedures' except that the gel-filtration step was omitted.

The relationship between inactivation and oxidation of cysteine residues was assessed by using a solution of galaptin that had been dialysed at 4 °C for 2.5 h against buffer A plus 0.1 mM-dithiothreitol and then 2.5 h against buffer A with continuous flushing with N_2. The final solution of galaptin (200 μg/ml) was kept at room temperature in the presence or absence of 1 mM-oxidized glutathione. For agglutination assays, 15 μl was diluted into 135 μl of buffer A plus 1 mg of bovine serum albumin/ml. For determination of protein thiol groups, a 200 μl sample was added to 50 μl of 150 mM-iodoacetamide in 1 M-Tris/HCl, pH 7.4; after 20 min, 225 μg of guanidinium chloride was added and the solution left for a further 1 h before dialysis, acid hydrolysis and amino acid analysis.

Alkylation of rat lung galaptin. The rates of the reactions with iodoacetamide or iodoacetate were generally determined at 4 °C with 20 mM-alkylating agent in 100 mM-Tris/HCl buffer (pH 7.8)/20 mM-sodium phosphate/140 mM-NaCl/5 mM-dithioerythritol. After designated times, the reaction was stopped by adding portions of the reaction mixture to 0.14 vol. of 10 mM-sodium phosphate buffer (pH 7.2)/70 mM-NaCl/2.5 mM-dithioerythritol/7 M-mercaptoethanol and used for haemagglutination assays and for amino acid analyses. One set of experiments was done with 250 mM-iodoacetate at room temperature.

Table 1. Amino acid composition of rat lung galaptin

The values for serine, threonine, cysteine and tryptophan were determined from different analyses (see under 'Methods'). As indicated previously (Whitney *et al.*, 1985), the composition reported earlier (Powell, 1980) was incorrect.

Amino acid	Composition (residues/subunit)
Asx	20.3
Thr	6.0
Ser	4.6
Glx	10.3
Pro	8.0
Gly	9.1
Ala	10.9
Cys	6.0
Val	5.5
Met	1.5
Ile	5.3
Leu	11.0
Tyr	0.8
Phe	9.1
His	2.9
Trp	0.9
Lys	8.2
Arg	3.9

Aminoethylation was accomplished by four additions of 0.01 vol. of ethyleneimine at 10 min intervals and at room temperature (**in a fume hood**) to rat lung galaptin in 400 mM-Tris/HCl buffer (pH 8.5)/4 mM-dithioerythritol. The modified protein was passed through a column of Bio-Gel A-0.5m and the protein peak taken for assay and amino acid analysis.

RESULTS

Amino acid composition of rat lung galaptin

The total number of oxidized plus reduced cysteine residues per subunit of rat lung galaptin was 5.8 by amino acid analysis after reaction of galaptin with iodoacetamide in the presence of dithiothreitol and guanidinium chloride. We found 6.0 cysteine residues in the reduced form after reaction in guanidinium chloride in the absence of reducing agents. We conclude that galaptin has six cysteine residues per subunit and that they are normally all in the reduced form under the conditions we use for purification, storage and assay.

The complete amino acid composition is shown in Table 1. It was determined using the above data for cysteine residues, 26 and 72 h hydrolyses to correct for losses of threonine and serine, and hydrolysis in 4 M-methanesulphonic acid to spare tryptophan. There is one tryptophan and one tyrosine residue per subunit, but the aspartic acid-plus-aspargine content is quite high.

Oxidative inactivation of human and rat galaptins

To assess how lung galaptin remains active in an extracellular environment, we assayed human fibroblast galaptin after incubation in tissue-culture flasks. We did not have enough fibroblast galaptin to use in the agglutination assay. Because it was radioactive, we used binding to a column of lactosyl-Sepharose and elution by lactose to measure activity. This assay probably overestimates activity, since dimeric galaptin molecules with only one functional binding site could still bind to lactosyl-Sepharose but would not be able to agglutinate red cells (see the Discussion section). The galaptin quickly lost its ability to bind lactosyl-Sepharose when incubated at 37 °C in fibroblast growth medium with or without 10% fetal-bovine serum (Table 2). Addition of 5 mM-GSH protected against the inactivation. However, if medium with GSH was incubated for 24 h under air/CO_2 (19:1) before adding galaptin, galaptin activity was lost within 1 h (Table 2). After adding 10 mM-dithioethreitol to the inactive galaptin and incubating at 4 °C for 16 h, 90% of the galaptin was re-activated, as assessed by its ability to bind to lactosyl-Sepharose (results not shown). In a simpler medium (buffer A with or without 0.3% bovine serum albumin) most of the activity survived at 37 °C for 1 h even without glutathione (Table 2). Addition of 10% fetal-calf serum to this medium accelerated the loss of activity; dialysis of the serum diminished, but did not eliminate, the accelerated loss of activity. GSH protected against this inactivation. In experiments without serum or serum albumin, less than 30% of the galaptin was recovered from the tissue-culture flasks, owing to adsorption of the protein to the plastic. Only the galaptin recovered from the flasks was included in activity calculations.

Rat lung galaptin was dialysed against buffer A under an N_2 atmosphere to remove dithiothreitol and maintain the protein thiols in the reduced form. The u.v. spectrum

Table 2. Oxidative inactivation of human lung fibroblast galaptin

Human fibroblast galaptin (1 μg) was incubated at 37 °C in 4 ml of medium in a T-25 plastic tissue-culture flask in CO_2/air (1:19). Glutathione was added in its reduced form (GSH). After incubation, activity of galaptin recovered from the flask was assessed by its binding to lactosyl-Sepharose (see the Experimental procedures section). Abbreviations used: EMEM, Eagle's minimal essential medium plus non-essential amino acids and Earle's salts; FCS, fetal-calf serum; Buffer A, 20 mM-sodium phosphate buffer (pH 7.2)/140 mM-NaCl; dial. FCS, FCS dialysed against buffer A; BSA, bovine serum albumin.

Medium	[GSH] (mM)	Incubation period (h)	Residual activity (%)
EMEM	0	1	4
	5	1	93
+10% FCS	0	1	3
	5	24	3
	5*	1	4
Buffer A	0	1	74
	5	1	79
+10% FCS	0	1	37
+10% FCS	5	1	86
+10% dial. FCS	0	1	49
+0.3% BSA	0	1	86

* EMEM+FCS+GSH were incubated at 37 °C for 24 h before galaptin was added.

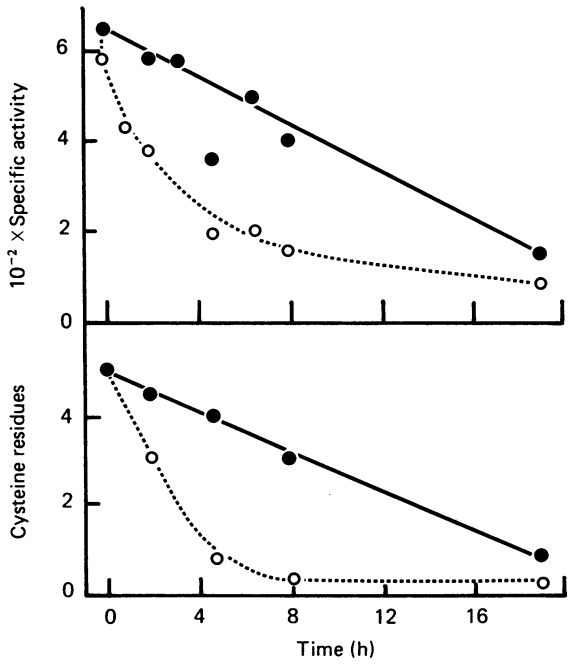

Fig. 2. Inactivation of rat lung galaptin by atmospheric oxygen

Galaptin in buffer A was exposed to air in the presence (○) or absence (●) of 1 mM-GSSG. Specific activity is expressed in units of haemagglutinating activity per mg of protein. 'Cysteine residues' is the number of reduced cysteine residues per subunit.

of the dialysed galaptin (Fig. 1) was the same as that obtained for galaptin dialysed against buffer with 5 mM-dithiothreitol in the presence or absence of lactose (results not shown). Dialysis to remove dithiothreitol and O_2 did not result in a significant change in specific agglutinating activity (970 units/mg using dithiothreitol-free titreing buffers). After the O_2-free galaptin solution was flushed with O_2, the u.v. spectrum (Fig. 1) was very similar to the initial spectrum, except that the later spectrum showed a small, general, increase in absorbance that was more pronounced at lower wavelengths; this change in spectrum may be due to increased light-scattering resulting from denaturation of a very small fraction of the galaptin and to oxidation of protein thiol groups. There is no evidence for oxidation of tryptophan; oxidation of tryptophan would have caused diminished absorbance at 280 nm and higher absorbance at 250 nm (Patchornik *et al.*, 1960). After 18 h the spectrum still had the same characteristic form, but the absorbance increase at low wavelengths was more pronounced. At this point the galaptin only had 0.3 thiol group remaining.

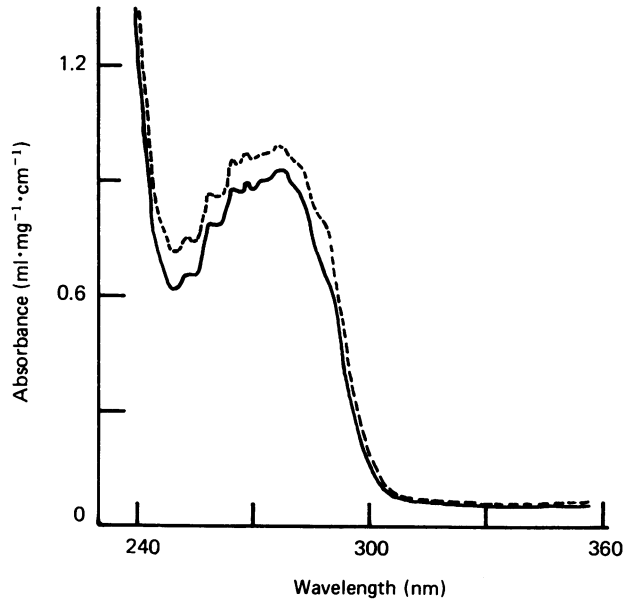

Fig. 1. Ultraviolet spectra of native and oxidized rat lung galaptin

Spectra for rat lung galaptin (350 μg/ml) in buffer A (——) and after flushing with O_2 (----) are shown.

The loss of activity and oxidation of protein thiol groups in rat lung galaptin were monitored by using a solution that was dialysed to remove O_2 and dithiothreitol and then exposed to air (Fig. 2). The half-times for loss of activity and oxidation of protein thiol groups were both about 11 h. When 1 mM-GSSG was included, loss of activity and oxidation of protein thiol groups were much more rapid; the half-times were about 2 h.

We attempted to investigate the effect of oxidation on the M_r values for galaptins from human lung fibroblasts and rat lungs by gel filtration (Fig. 3). The oxidized galaptins were eluted later than was the native, reduced, form. In the experiment with partially oxidized rat lung galaptin, agglutinating activity was detected at the shoulder at 52 ml, but not at the peak of 55 ml. The delayed elution could reflect increased non-specific

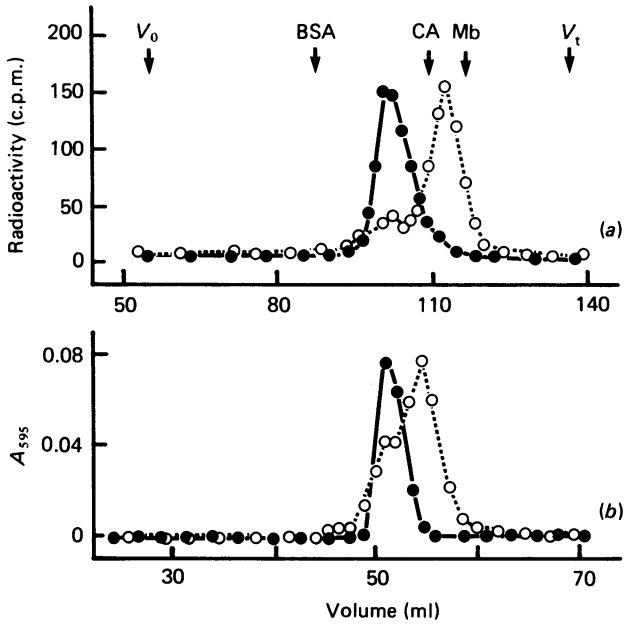

Fig. 3. Gel filtration of native and oxidized galaptins

Chromatography was done at 4 °C with buffer A plus 10 mM-lactose and 5 mM-dithiothreitol (for native galaptins) or buffer A plus 10 mM-lactose (for oxidized samples). (*a*) A column (1.5 cm × 88 cm) of Bio-Gel A-0.5m was calibrated with Blue Dextran (V_0), bovine serum albumin (BSA), M_r 66000, human carbonic anhydrase II (CA), M_r 29000, sperm-whale myoglobin (Mb), M_r 17000, and NaCl (V_t). The two samples analysed were radioactive galaptin, in its reduced form (——), purified from human lung fibroblasts (see the Experimental procedures section) and an oxidized sample (----) further treated by incubation at 37 °C in Eagle's minimal essential medium for 1 h (see Table 2). (*b*) The column of Bio-Gel A-0.5m was 1.5 cm × 41 cm. The samples were rat lung galaptin (70 μg in 1 ml) in its reduced form (——) and an oxidized sample (----) incubated at room temperature in air in the absence of exogenous thiols for 20 h before chromatography.

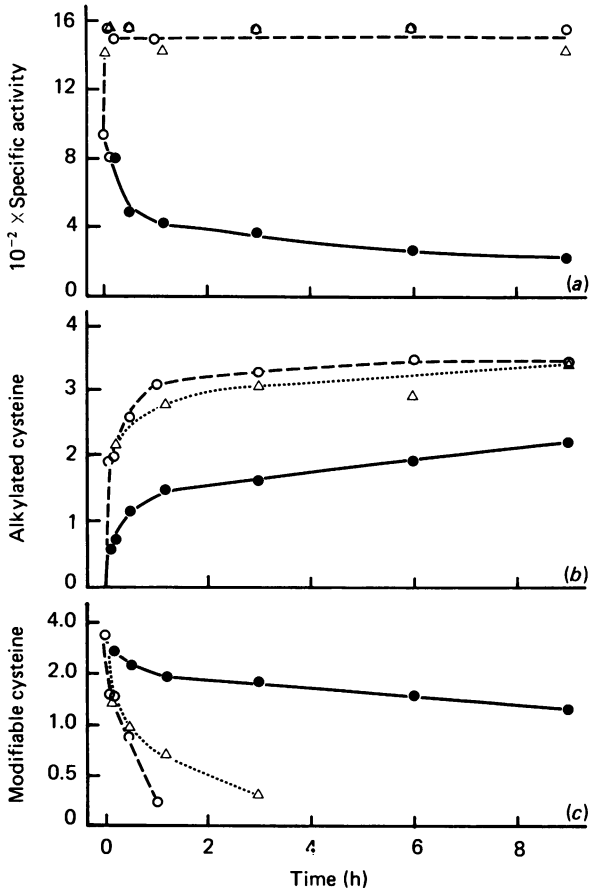

Fig. 4. Alkylation of rat lung galaptin

Galaptin was allowed to react at 4 °C with 20 mM-iodoacetamide (○--○), 20 mM-iodoacetamide plus 10 mM-lactose (△····△) or 20 mM-iodoacetate (●——●). After designated times the reaction was quenched by adding excess mercaptoethanol. (*a*) Specific haemagglutinating activity is expressed in units/mg of protein. The dotted line follows the same course as the broken line. (*b*) 'Alkylated cysteine' means number of residues of alkylated cysteine per subunit of galaptin. (*c*) First-order semilogarithmic plot of number of modifiable cysteine residues remaining ('modifiable cysteine'), assuming a total of 3.5 modifiable cysteine residues per subunit (based on the results in *b*).

association of galaptin with the gel matrix, a contraction in molecular size, or dissociation of the native, dimeric, lectin to monomers. Sedimentation-equilibrium experiments with rat lung galaptin showed that the M_r values for the native (26800 ± 1800) and oxidized (25100 ± 1300) galaptins were not significantly different. Neither protein showed a significant change in apparent M_r over a 4-fold range in protein concentration. Plots of the logarithm of concentration against the square of the distance from the centre of the rotor showed no significant deviation from linearity. The lowest galaptin concentration used in the ultracentrifuge was three times higher than that used for gel filtration. However, since the experimental points in the plot from sedimentation equilibrium were linear even through the region of low protein concentration at the top of the cell, this suggests that galaptin was not dissociating to a significant extent. These observations support the conclusion that oxidation did not cause the dimeric galaptin to dissociate or aggregate to a significant extent.

Oxidation did not result in disulphide bonding between subunits. Native and oxidized rat lung galaptins were electrophoresed in polyacrylamide gels in the presence of sodium dodecyl sulphate in the absence of reducing agents. Both preparations showed only the monomer.

Alkylation of rat lung galaptin

Not all of the six thiol groups in a subunit of rat lung galaptin are freely accessible for reaction in native galaptin. Iodoacetamide reacted with two cysteine residues per subunit in native galaptin within 0.1 h (Fig. 4); this was accompanied by a 60% increase in specific activity over that found for native galaptin. By 3 h, 1.5 more thiol groups had reacted at a somewhat lower rate. No further reaction took place between 3 and 9 h, leaving two to three thiol groups that did not react. After the first 10 min there was no further change in activity. Lactose did not appear to alter the extent of reaction or

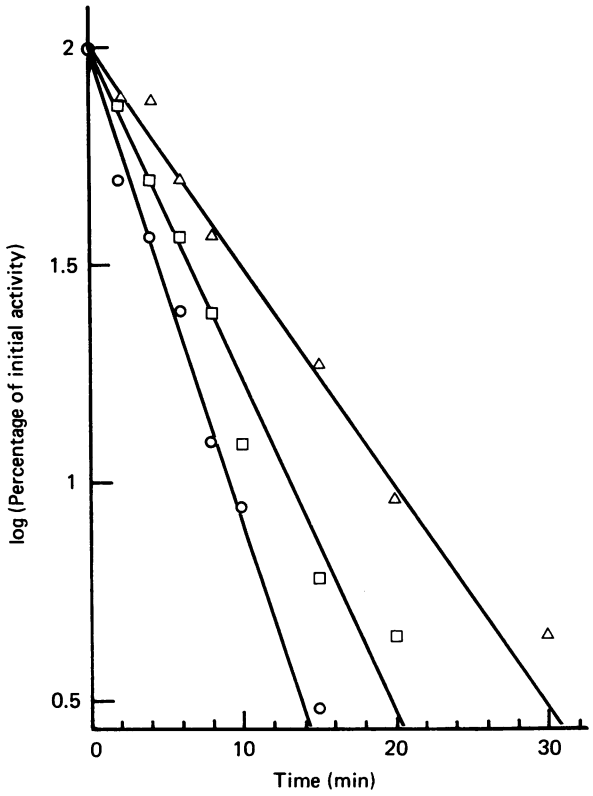

Fig. 5. Inactivation of rat lung galaptin by iodoacetate

Galaptin was allowed to react at room temperature with 250 mM-iodoacetate in the absence of hapten (○), in the presence of 5 mM-lactose (□) or in the presence of 0.3 mM-lacto-*N*-tetraose (△). The 'initial activity' refers to haemagglutination activity of the galaptin in buffer with 2 mM-mercaptoethanol.

the increase in activity that occurred during the first 0.1 h, but the reaction was too rapid for a definitive conclusion from these results. Lactose did retard the rate of reaction with the slower-reacting thiol groups (Fig. 4).

The Cam-galaptin prepared by reaction with native galaptin behaves as a dimer during gel filtration through Bio-Gel A-0.5m. It also retains its ability to bind to lactosyl-Sepharose. The binding activity and the haemagglutinating activity are stable in the absence of reducing agents for several days at 4 °C. Since Cam-galaptin still has two thiol groups per subunit, we included 5 mM-dithiothreitol in the buffer for long-term storage. We have stored Cam-galaptin this way for 3 months with no loss of activity. Batches of Cam-galaptin are readily prepared by allowing galaptin to react with 25 mM-iodoacetamide at 4 °C for 3 h in a buffered solution (pH 7.2) containing 5 mM-dithiothreitol or for 9 h (or overnight) if the solution also contains 10 mM-lactose.

Iodoacetate reacted much more slowly than iodoacetamide, and iodoacetate caused the activity to diminish rather than increase. Only one thiol group per subunit reacted fairly rapidly ($t_{\frac{1}{2}}$ 0.2 h), two reacted slowly (3.3 groups had reacted after 27 h) and the rest were essentially unreactive. Analysis of fractions collected during amino acid analysis of Cm-galaptin prepared by reaction with iodo[^{14}C]acetate for 6 h showed that only thiol groups were alkylated. Iodoacetate apparently reacted with the same thiol groups as did iodoacetamide, since iodoacetate did not react with Cam-galaptin. Cm-galaptin also behaves as a dimer during gel filtration through Bio-Gel A-0.5m and binds to lactosyl-Sepharose. Loss of activity was associated with carboxymethylation of rapid- and slow-reacting thiol groups.

Galaptin haptens retarded the loss of activity resulting from reaction with 250 mM-iodoacetate at room temperature (Fig. 5) The half-time of reaction ($t_{\frac{1}{2}}$ 7 min) increased to 12 min in the presence of 5 mM-lactose and to 15 min with 0.3 mM-lacto-*N*-tetraose; the concentrations of lactose and lacto-*N*-tetraose required to effect 50% inhibition of haemagglutination by galaptin were 0.3 mM and 0.07 mM respectively.

We also examined galaptin after alkylation of all six thiol groups under denaturing conditions. After reaction with iodoacetamide or iodoacetate and removal of the guanidinium chloride by dialysis, the alkylated galaptins appeared to be dimers on the basis of their Bio-Gel A-0.5m gel-filtration behaviour. In the haemagglutination assay the carboxymethylated galaptin was inactive, but the carboxamidomethylated galaptin retained 20% of the activity of the native galaptin.

Aminoethylation of galaptin led to loss of 85% of the haemagglutination activity, but the modified protein still bound to lactosyl-Sepharose. After four additions of ethyleneimine, we found 2.4 residues of aminoethylcysteine by amino acid analysis. Gel filtration through Bio-Gel A-0.5m indicated that the aminoethylated galaptin remained a dimer. The residual activity was stable in the absence of reducing agents. Ethyleneimine is no longer available (it is a carcinogen), so we have not followed up these studies. Since the reactivity of protein thiol groups is influenced greatly by protein structure, a substitute such as *N*-(iodoethyl)trifluoroacetamide would not be expected to have the same reaction pattern as ethyleneimine.

DISCUSSION

Vertebrate galaptins are inactivated by atmospheric oxygen in the absence of exogenous thiols (Barondes, 1984). Rat lung galaptin is a relatively small protein (a dimer of 13.5 kDa subunits) that is rich in cysteine residues (six per subunit), but has only one tryptophan residue per subunit. The electric organ of *Electrophorus electricus* contains electrolectin, a β-galactoside-specific lectin similar to lung galaptin, that is inactivated by oxygen and protected by thiol reagents (Levi & Teichberg, 1981). Inactivation of electrolectin was linked to oxidation of the tryptophan side chain to form an oxindole derivative. The mechanism for oxidative inactivation of rat lung galaptin, on the other hand, is oxidation of cysteine residues; inactivation parallels oxidation of cysteine residues, whereas the tryptophan residue remains unaltered. It is interesting that both proteins are inactivated by atmospheric oxygen but by such different mechanisms.

The rate of oxidative inactivation varies considerably under different experimental conditions. In culture medium at 37 °C, dilute human galaptin is more than 90% inactivated within 1 h, but it loses less than 15% activity in a simple buffer supplemented with 0.3% bovine serum albumin. GSSG accelerated the inactivation of rat lung galaptin, probably by catalysing intramolecular protein disulphide-bond formation

and/or forming mixed disulphides between protein and glutathione (*S*-thiolation; see Ziegler, 1985).

In the above experiments with human galaptin, its activity was assessed by its binding to a column of lactosyl-Sepharose followed by elution with lactose. There are two ways in which this binding assay is less sensitive than the agglutination assay in measuring changes in galaptin function. Firstly, haemagglutination activity requires that both subunits have a functional binding site, but binding to lactosyl-Sepharose requires only a single functional binding site per dimer. Secondly, the haemagglutination assay is more sensitive to changes in galaptin–sugar affinity. We have previously shown that only about 20% of the galaptin is bound to erythrocytes at the haemagglutination titre end point (Whitney *et al.*, 1985), so a decreased affinity of galaptin for the erythrocytes would necessitate the use of higher galaptin concentrations to bring about agglutination. In terms of units of agglutination activity, a decrease in binding affinity would have the same effect as total inactivation of a fraction of the galaptin molecules. On the other hand, binding of native galaptin to lactosyl-Sepharose is strong enough for the galaptin to be still completely retained by the column even if there is a fairly large decrease in binding affinity. Therefore, galaptin molecules with only one functional site, or with sites with somewhat lower affinity, will show decreased agglutination activity but still retain full activity in the lactosyl-Sepharose binding assay. These considerations lead us to conclude that the agglutination assay would have shown even more rapid loss of function.

All the cysteine residues in native rat lung galaptin are in the reduced form, with no evidence of any disulphide bonds. Oxidation of the protein thiol groups to disulphide bonds by atmospheric oxygen does not result in intersubunit disulphide-bond formation or an increase in M_r. Some protein conformational rearrangements probably occur during the process of disulphide-bond formation; the retarded elution of oxidized galaptin from the gel-filtration column suggests that the conformational changes result in a more compact structure or in a structure that has higher non-specific affinity for the gel matrix. The loss of galaptin activity is probably also a result of conformational changes and not due to a direct, critical, function of a cysteine residue in the carbohydrate-binding site.

The native galaptin structure places the different cysteine residues in a variety of environments with different accessibilities to solvent components. This leads to a wide range in rates at which the thiol groups are alkylated by iodoacetate and iodoacetamide; two thiol groups per subunit are modified by iodoacetamide within 10 min, one to two more react within 3 h and the rest remain unmodified in 9 h. The unreactive ones are most likely buried within the protein molecule. The protection afforded by galaptin inhibitors may be due to stabilization of the native protein structure that provides shielding from reagents in solution rather than to direct steric hindrance by the bound inhibitor.

The stability of Cam-galaptin in the presence of oxygen has proved to be useful to us. We have used it to prepare an affinity column for endogenous ligands of lung galaptins (Powell & Whitney, 1984), measure the binding of galaptin to isolated lung cells (Whitney *et al.*, 1985), and prepare antibodies to galaptin (J. T. Powell, P. L. Whitney & L. B. Clerch, unpublished work).

Are there important specific functions for some of these cysteine residues? The reactive thiol groups are not likely to be directly involved in carbohydrate binding, but it is clear that some of the thiol groups must remain in their reduced form to maintain galaptin in its active form. One problem in trying to decide if the thiol groups have a functional role is that we still know little of the function of galaptin, except that it binds β-galactosides and is able to aggregate trypsin-treated rabbit red blood cells; the latter activity is almost certainly not a physiological one. If galaptin has an extracellular function then the lability of its binding activity in the presence of oxygen could put stringent restrictions on the latitude of its activity. This could be an important advantage if only a transient function is needed. Another possibility is that galaptin could be linked to other macromolecular components via disulphide bonding.

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REFERENCES

Barondes, S. H. (1981) Annu. Rev. Biochem. **50**, 207–231
Barondes, S. H. (1984) Science **223**, 1259–1264
Barondes, S. H. & Haywood-Reid, P. L. (1981) J. Cell Biol. **91**, 568–572
Beyer, E. C. & Barondes, S. H. (1982) J. Cell Biol. **92**, 23–27
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Cecil, R. (1963) Proteins **1**, 379–476
Cerra, R. F., Haywood-Reid, P. L. & Barondes, S. H. (1984) J. Cell Biol. **98**, 1580–1589
Cohn, E. J. & Edsall, J. T. (1943) Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, p. 375, Hafner Publishing Co., New York
Eagle, H. (1959) Science **130**, 432–437
Earle, W. R. (1943) J. Natl. Cancer Inst. **4**, 165
Harrison, F. L. & Chesterton, C. J. (1980) FEBS Lett. **122**, 157–165
Kobata, A. (1972) Methods Enzymol. **25**, 262–270
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Levi, A. & Teichberg, V. I. (1981) J. Biol. Chem. **256**, 5737–5740
Levi, A. & Teichberg, V. I. (1985) Biochem. J. **226**, 379–384
Patchornik, A., Lawson, W. B., Gross, E. & Witkop, B. (1960) J. Am. Chem. Soc. **82**, 5923–5927
Podleski, T. R. & Greenberg, I. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1054–1058
Powell, J. T. (1980) Biochem. J. **187**, 123–129
Powell, J. T. & Whitney, P. L. (1984) Biochem. J. **223**, 769–774
Sanford, G. L., Davis, L. D. & Powell, J. T. (1982) Biochem. J. **204**, 97–102
Whitney, P., Maxwell, S., Ryan, U. & Massaro, D. (1985) Am. J. Physiol. **284** (Cell Physiol. **17**), C258–C264
Wrigley, C. W. (1971) Methods Enzymol. **22**, 559–564
Yphantis, D. A. (1964) Biochemistry **3**, 297–317
Ziegler, D. M. (1985) Annu. Rev. Biochem. **54**, 305–329

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