The Binding and Catalytic Activities of Forms of Ligandin after Modification of its Thiol Groups

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Ligandin (glutathione *S*-transferase B, EC 2.5.1.18) was treated with *p*-mercuribenzoate, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, 5,5′-dithiobis-(2-nitrobenzoic acid), *N*-ethylmaleimide, iodoacetamide or iodoacetate. Although performic acid oxidation revealed the presence of four cysteines, *p*-mercuribenzoate and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, the most effective of the reagents studied, reacted with only three residues. *N*-Ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) each reacted with two cysteines: iodoacetamide reacted with only one cysteine and iodoacetate was essentially unreactive. Modification of three thiol groups decreased both the enzymic and binding activities of ligandin although the number of binding sites was unaffected. Modification of only one or two of the thiol groups had little effect on the ligandin activities. It therefore appears that there is a thiol group in the common hydrophobic-ligand- and substrate-binding site of ligandin. Ligandin was separated into two fractions on CM-cellulose. Both fractions gave the same results with *p*-mercuribenzoate and iodoacetamide.

Ligandin is a soluble protein from the rat occurring in a variety of tissues (Litwack *et al.*, 1971; Bannikov & Tchipesheva, 1972; Bass *et al.*, 1977) and particularly abundant in the liver (Litwack *et al.*, 1971; Fleischner *et al.*, 1972; Bass *et al.*, 1977). Similar proteins have been found in other species including man (Trip *et al.*, 1974; Habig *et al.*, 1976; Asaoka *et al.*, 1977). It is a basic protein (Litwack *et al.*, 1971; Ketterer *et al.*, 1967) which is microheterogeneous according to several criteria (Ketterer *et al.*, 1967, 1975; Listowsky *et al.*, 1976; Bass *et al.*, 1977).

Ligandin binds a number of compounds with hydrophobic moieties, both exogenous and endogenous, at a single site (Litwack *et al.*, 1971; Kamasaka *et al.*, 1975; Ketterer *et al.*, 1975; Ketley *et al.*, 1975; Tipping *et al.*, 1976*a,b,c*, 1978). It also binds glutathione in an adjacent site (Tipping *et al.*, 1976*c*; Jakoby, 1978). This latter binding site confers certain enzymic activities on ligandin including glutathione *S*-transferase (EC 2.5.1.18) (Habig *et al.*, 1974*a*), 3-oxo steroid Δ^4–Δ^5-isomerase (Benson *et al.*, 1977), 'nitrate reductase' (Habig *et al.*, 1975) and glutathione peroxidase (Proshaska & Ganther, 1977). In the case of glutathione *S*-transferase activity, hydrophobic ligands compete with electrophilic substrates (Ketterer *et al.*, 1976; Jakoby *et al.*, 1976).

As a result of its binding and catalytic properties involving glutathione conjugation, it has been pro-

posed that ligandin may play an important role in intracellular transport and detoxification (Jakoby & Keen, 1977; Meuwissen *et al.*, 1977).

Previously it has been shown that aminoazo-dye carcinogens bind non-covalently to purified ligandin (Ketterer *et al.*, 1976) and also covalently *in vivo* (Ketterer & Christodoulides, 1969/1970). Covalent binding is presumed to result from reaction with a metabolite of the azo dye that is electrophilic and therefore capable of reaction with a suitably placed nucleophilic side chain at the binding site. This electrophile may be the sulphate ester of *N*-hydroxy-*N*-methyl-4-aminoazobenzene, which is believed to be the ultimate carcinogen (Kadlubar *et al.*, 1976). Since binding occurs to cysteine residues (Ketterer & Christodoulides, 1969/1970) it is possible that thiol groups are involved in the binding and catalytic sites of ligandin. This possibility has been investigated by determining the effect of various modifications of thiol groups of ligandin on its binding and catalytic activities. The binding of oestrone sulphate and bilirubin was used to indicate the effect of modification on the binding properties of ligandin, and glutathione *S*-transferase and 3-oxo steriod Δ^4–Δ^5-isomerase activities were used as an indication of the effect of modification on catalytic activity.

A modification of the procedure for isolation of ligandin enabled its separation into a major and a minor fraction differing in their behaviour on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The thiol groups of both of these fractions have been studied.

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Experimental

Materials

Oestrone sulphate (potassium salt), dithiothreitol, glutathione (reduced) and bilirubin were from Sigma Chemical Co. (Poole, Dorset, U.K.), as were p-hydroxymercuribenzoate, iodoacetate, iodoacetamide, 5,5'-dithiobis-(2-nitrobenzoic acid) and N-ethylmaleimide. N-(4-Dimethylamino-3,5-dinitrophenyl)-maleimide was supplied by Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Sodium sulphobromophthalein was purchased as a 5% aqueous suspension from Hyman, Westcott and Dunning Inc. (Baltimore, MD, U.S.A.). Sephadex G-100 and CM-Sephadex C-50 were supplied by Pharmacia (London W.5, U.K.). DEAE-cellulose (DE-23) and CM-cellulose (CM-52) were products of Whatman (Springfield Mill, Maidstone, Kent, U.K.). Ampholines were from LKB Instruments (Croydon, Surrey, U.K.). Radioactively labelled oestrone sulphate was from The Radiochemical Centre (Amersham, Bucks., U.K.) and labelled glutathione was from New England Nuclear (Penicuik, Midlothian, Scotland, U.K.).

Methods

Fractionation of ligandin. Ligandin was prepared by the method of Tipping et al. (1976a) and fractionated by application to a CM-cellulose column (2.6cm×30cm) equilibrated with 10mM-potassium phosphate, pH6.7, and eluted with a linear gradient of 0–0.2M-KCl in the same buffer. Fractions of ligandin purified in this manner were either used immediately or dialysed against water, freeze-dried and stored at −20°C.

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin [$A_{279}^{1\%} = 6.7$ (Reynolds et al., 1967)] as standard.

Binding studies. Bilirubin-binding measurements based on the difference spectrum observed on the binding of bilirubin to ligandin were made by the method of Tipping et al. (1976a). The difference spectrum has a peak near 470nm and a trough near 400nm. To increase the sensitivity of the assay the difference between the trough and the peak (ΔA_{470}–ΔA_{400}) was used in place of the difference absorbance of the peak (ΔA_{470}) only.

Oestrone sulphate binding was measured by equilibrium dialysis as described by Tipping et al. (1976c).

Enzyme activities. The glutathione S-transferase activity of ligandin was assayed by the method of Habig et al. (1974b) with 1-chloro-2,4-dinitrobenzene as substrate. 3-Oxo steroid Δ^4–Δ^5-isomerase activity was measured by the method of Benson et al. (1977).

Reaction with p-mercuribenzoate. Ligandin was titrated with p-mercuribenzoate and the reaction followed spectrophotometrically by the method of

Boyer (1954). The titration was performed in 0.05M-sodium phosphate buffer, pH7.0, in preparation for equilibrium dialysis with oestrone sulphate or in 0.1M-Tris/HCl buffer, pH8.2, in preparation for binding studies with bilirubin. The effect of the modification on enzymic activity was determined at both pH values.

Reaction with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide. Ligandin was treated with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide by the method of Witter & Tuppy (1960). Preliminary experiments were done in 0.33M-sodium acetate buffer, pH4.6, for 1h. However, this pH recommended by Witter & Tuppy (1960) caused denaturation of ligandin, and although it appeared to be reversible, it was considered prudent to carry out the experiment at pH7.0 in 0.05M-phosphate buffer, with a 30-fold excess of reagent to ensure complete reaction.

Enzyme assays were performed on the undialysed reaction mixture, an equal amount of the thiol reagent being added to the assays of the controls. Excess reagent was removed by dialysis before both binding studies and titration with p-mercuribenzoate (as above) to measure unchanged groups.

Reaction with iodoacetate and iodoacetamide. Ligandin was treated with iodoacetate and iodoacetamide in 0.05M-sodium phosphate buffer, pH8.0. The concentration of the reagent was varied between 5 and 100mM. Reaction times were generally 4h and the solutions were thoroughly flushed with N_2 and shielded from light with aluminum foil. Small samples were removed for the determination of enzymic activities. The cysteine groups which had reacted were estimated as carboxymethylcysteine in samples which had been dialysed, freeze-dried, hydrolysed in 6M-HCl and subjected to amino acid analysis.

In another series of experiments ligandin was treated with 0.17M-iodoacetamide in 8M-urea and in the presence and absence of 0.17M-mercaptoethanol or without urea, again in the presence and absence of mercaptoethanol. Carboxymethylcysteine was determined by amino acid analysis as described above (Crestfield et al., 1963).

Reaction with N-ethylmaleimide. Ligandin was treated with 0.1M-N-ethylmaleimide in 0.05M-sodium phosphate buffer, pH7.0, and the reaction followed by the decrease in A_{302} (Colman & Chu, 1970). Samples were removed at various times, diluted in assay buffer to stop the reaction and glutathione S-transferase and 3-oxo steroid Δ^4–Δ^5-isomerase activities determined.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) was by the method of Ellman (1959). The reaction was carried out in 0.05M-phosphate buffer, pH7.0, and followed by the increase in A_{412}. The thiol reagent was added to give a concentration of 50μM.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed using the Tris/glycine buffer system outlined by Maizel (1971). An acrylamide:bisacrylamide ratio of 30:0.8 was used with a 10% resolving and 3% spacer gel. The gels contained 8M-urea and 0.1% sodium dodecyl sulphate. The electrode buffer also contained 0.1% sodium dodecyl sulphate.

Samples were prepared by heating to 100°C for several minutes in 8M-urea, 1% sodium dodecyl sulphate and 0.1% mercaptoethanol.

Electrophoresis was done on a Raven standard model slab-gel apparatus (Raven Instruments, Haverhill, U.K.). Gels were stained with 0.5% Amido Black in an aqueous solution of 20% methanol and 2.2% acetic acid. They were destained by diffusion in 7.5% acetic acid/35% ethanol.

Circular dichroism. Measurements were made with a Cary 61 spectropolarimeter (Varian Instruments Division, Palo Alto, CA, U.S.A.) at 24°C in 1 cm and 0.1 cm fused-quartz cells.

Spectrophotometry. Spectrophotometric measurements were made with a Pye–Unicam SP.1800 double-beam spectrophotometer (Pye Instruments, Cambridge, U.K.) in silica cells of 0.1, 1.0 and 4.0 cm path-length.

Amino acid analysis. Amino acid analyses were performed on a JEOL JLC 6AH amino acid analyser (JEOL Instruments, Tokyo, Japan) by the method of Crestfield *et al.* (1963). All samples containing carboxymethylcysteine were thoroughly flushed with N_2 and evacuated before being sealed for hydrolysis. Samples were hydrolysed in 6M-HCl for 24 h at 105°C.

Performic acid oxidations were done as outlined by Hirs (1967). The oxidations were left to proceed overnight at 0°C.

Results

Fractionation of ligandin

Ligandin separated into two peaks on CM-cellulose eluted with a salt gradient (Fig. 1). The first peak (fraction 1) showed a single band on polyacrylamide-gel electrophoresis corresponding to an apparent mol.wt. of 22000, whereas the second peak (fraction 2) showed two bands corresponding to apparent mol. wts. of 22000 and 25000 (Fig. 2). Most experiments were performed on fraction 1, but the effects of *p*-mercuribenzoate and iodoacetamide on fraction 2 were also determined.
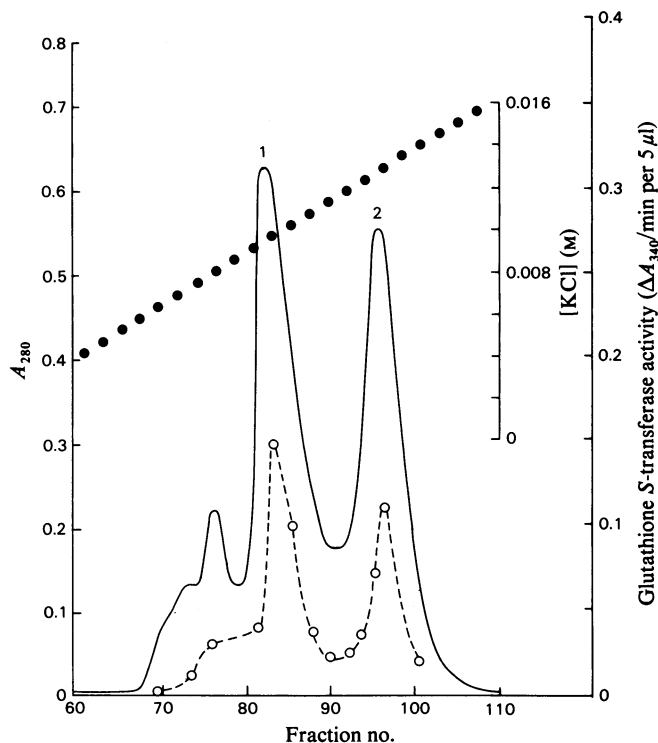

Fig. 1. *Fractionation of ligandin on CM-cellulose*
———, A_{280}; ----, glutathione *S*-transferase activity; ●, KCl gradient.

Amino acid analysis

There was no statistically significant difference in the amino acid composition of fractions 1 and 2. The cysteine content of fraction 1 determined as cysteic acid was found to be 4.0. The cysteine content of fraction 2 was 3.7.

Reaction with p-mercuribenzoate

Typical titration curves for fraction 1 ligandin with p-mercuribenzoate at pH 7.0 and 8.2 are shown in Figs 3(a) and 3(b). p-Mercuribenzoate reacted with 3.2 thiol groups per ligandin molecule and decreased the glutathione S-transferase activity to 10–20% of the control activity and the 3-oxo steroid $\Delta^4-\Delta^5$-isomerase activity to 30–40% of the control. During the titration at pH 7.0, the loss of enzymic activity paralleled the reaction of p-mercuribenzoate with the thiol groups. At pH 8.2, however, the activity loss did not parallel the reaction with the thiol groups, no activity being lost until the titration of the third group. At either pH the loss of activity was greater for
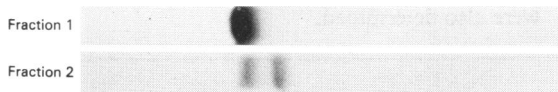

Fraction 1

Fraction 2

Fig. 2. *Polyacrylamide-gel electrophoresis of fractions 1 and 2*

A_{250}

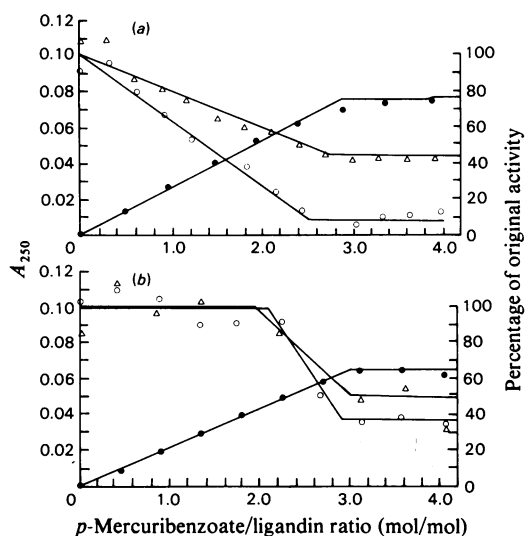p-Mercuribenzoate/ligandin ratio (mol/mol)

Fig. 3. *Titration of ligandin with p-mercuribenzoate* (a) Reaction at 7.0; (b) reaction at pH 8.2 ●, Reaction with p-mercuribenzoate indicated by A_{250}; ○, glutathione S-transferase activity; △, 3-oxo steroid Δ^4—Δ^5-isomerase activity.

the glutathione S-transferase activity than for the 3-oxo steroid $\Delta^4-\Delta^5$-isomerase activity.

The effect of p-mercuribenzoate on the binding of oestrone sulphate was determined by equilibrium dialysis and the results were analysed by means of Scatchard plots. The p-mercuribenzoate treatment, decreased the association constant from 3.5×10^5 to 1.2×10^5, but did not significantly decrease the number of binding sites. In each case a single binding site for oestrone sulphate was found, in agreement with the results of Tipping *et al.* (1976c).

The binding of bilirubin to native and p-mercuribenzoate-modified ligandin was also analysed by means of Scatchard plots, and p-mercuribenzoate showed a similar effect. The binding constant was decreased from 6.0×10^6 to 0.9×10^6 and again the number of binding sites was unaffected.

The visible difference spectrum resulting from the binding of bilirubin to ligandin can be used as an indication of binding. As shown in Fig. 4(a), modi-
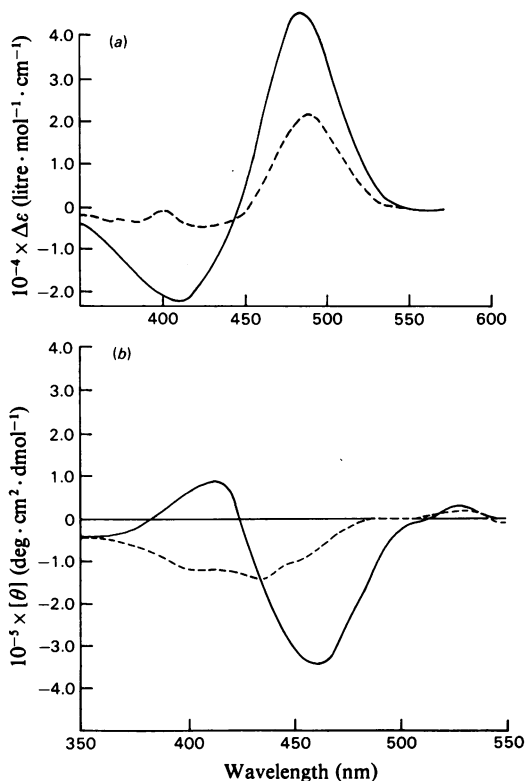

$10^{-4} \times \Delta\epsilon$ (litre·mol^{-1}·cm^{-1})

$10^{-5} \times [\theta]$ (deg·cm^2·dmol^{-1})

Wavelength (nm)

Fig. 4. *Effect of p-mercuribenzoate modification on the visible difference and circular dichroism spectra of ligandin* (a) Visible difference spectrum; (b) circular-dichroism spectrum (based on bound bilirubin). ——, Spectra obtained with native ligandin; ----, spectra obtained with ligandin modified with p-mercuribenzoate

fication of ligandin with p-mercuribenzoate decreases the intensity of the difference spectrum and causes a slight red shift. Reaction with p-mercuribenzoate also caused a marked decrease in intensity of the circular-dichroism spectrum as shown in Fig. 4(b).

The pH 7.0 titration was repeated in the presence of 0.1 mM-oestrone sulphate and 1 mM-sulphobromophthalein to determine if ligands protect one or more of the thiol groups from reaction. Oestrone sulphate had no effect on the number of groups reacting with p-mercuribenzoate and did not prevent the concomitant loss of glutathione S-transferase and 3-oxo steroid Δ^4-Δ^5-isomerase activities. Sulphobromophthalein binds much more strongly to ligandin than does oestrone sulphate (Tipping et al. 1976c) and prevented the loss of both the glutathione S-transferase and 3-oxo steroid Δ^4-Δ^5-isomerase activities. As sulphobromophthalein absorbs strongly at 250 nm, the titration with p-mercuribenzoate could not be followed.

Fraction-2 ligandin gave the same results with p-mercuribenzoate.

Reaction with N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide

At pH 4.6 all of the cysteine residues that react with p-mercuribenzoate also reacted with N-(dimethylamino-3,5-dinitrophenyl)maleimide, as no groups titratable with p-mercuribenzoate remained after reaction with a 6-fold excess of reagent. After leaving for several minutes in assay buffer to reverse the denaturation caused by the low pH it appeared that both the glutathione S-transferase and 3-oxo steroid Δ^4-Δ^5-isomerase activities were decreased to 30% of the control. A titration with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide at pH 7.0 to a 6-fold excess showed a similar reactivity of the thiol groups and effect on enzymic activities, although the enzymic activities were only decreased to 40% of the controls.

All further binding and enzymic activities were performed on ligandin treated with a 30-fold excess of reagent at pH 7.0. Under these conditions glutathione S-transferase activity was decreased to 45% of the control and the 3-oxo steroid Δ^4-Δ^5-isomerase activity was decreased to 20% of the control. This differs from the reaction with p-mercuribenzoate, which had a greater effect on the glutathione S-transferase activity than on the 3-oxo steroid Δ^4-Δ^5-isomerase activity.

The effect of N-(4-dimethylamino-3,5-dinitrophenyl) maleimide on the binding of ligands to ligandin was examined by the effect of the modification of the visible difference and circular-dichroism spectra of bilirubin bound to ligandin. As with the reaction with p-mercuribenzoate, the reaction of thiol groups with N-(4-dimethylamino-3,5-dinitro-

phenyl)maleimide resulted in a marked decrease in the intensity of both spectra.

N-(4-Dimethylamino-3,5-dinitrophenyl) maleimide-modified ligandin was also used to determine the effect of the modification of thiol groups on the binding of glutathione. This was accomplished by equilibrium dialysis by the method outlined for oestrone sulphate and glutathione concentrations between 10 and 200 μM. Dithiothreitol (1 mM) was added to all solutions to ensure that all of the glutathione present was reduced. The association constant was decreased slightly from 9.9×10^4 to 8.0×10^4 by N-(4-dimethylamino-3,5-dinitrophenyl)maleimide treatment.

If N-(4-dimethylamino-3,5-dinitrophenyl)maleimide was added to the assay solution for glutathione S-transferase or 3-oxo steroid Δ^4-Δ^5-isomerase activities an inhibitory effect was observed, which could not be explained in terms of reaction with ligandin, making it necessary to add the reagent to the control assay as well. Provided that the amount of reagent added was small the effect on the assay was only of the order of 5%. The inhibition is presumed to be to the formation of the glutathione adduct of the thiol reagent. It is thought that such an adduct would be an inhibitor, since glutathione adducts bind to ligandin (Tipping et al., 1976c) and are inhibitors of glutathione S-transferase A (Jakoby et al., 1976).

Reaction with iodoacetate and iodoacetamide

Iodoacetate did not react with ligandin at a concentration of 5 mM and only 0.3 group/molecule reacted with 100 mM-iodoactate. No loss of activity was observed in either case. On the other hand, 0.9 group/molecule was found to react with 5 mM-iodoacetamide. No further reaction was observed in the absence of denaturants using concentrations of iodoacetamide up to 100 mM. The reaction with iodoacetamide did not decrease either of the enzymic activities studied. Iodoacetamide also failed to show any significant effect on the difference spectrum of bilirubin on binding to ligandin.

When treated with 0.17 M-iodoacetamide in the presence of 8 M-urea and 0.17 M-mercaptoethanol, 3.1 mol of carboxymethylcysteine was formed per mol of ligandin. It was found that mercaptoethanol was not required for maximal reaction of the cysteine residues and its addition to ligandin in the absence of denaturant did not result in the modification of more than one thiol group.

Similar results were obtained with fraction-2 ligandin.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid)

5,5'-Dithiobis-(2-nitrobenzoic acid) reacted with 2.0 thiol groups/molecule reducing the glutathione S-transferase activity by 20%. This reaction also

had only a small effect on the binding of bilirubin, the intensity of the peak of the difference spectrum was decreased by only 19%.

Reaction with N-ethylmaleimide

N-Ethylmaleimide also reacted with 2.0 thiol groups/molecule, but this was not accompanied by any decrease in glutathione S-transferase or 3-oxo steroid Δ^4-Δ^5-isomerase activities.

Discussion

These results indicate that ligandin has a total of four thiol residues. The lack of effect of mercapto-ethanol on reaction with iodoacetamide in 8M-urea suggests that all of the thiol groups are present as free cysteine.

The reactivity of the thiol residues varies with the reagent. Iodoacetamide reacts with only one residue and iodoacetate is even less reactive. N-ethylmale-imide and 5,5'-dithiobis-(2-nitrobenzoic acid) each react with 2 mol of cysteine per mol of ligandin. The next most effective is p-mercuribenzoate, which reacts with three cysteine residues; the fourth is apparently inaccessible to iodoacetamide in the presence of 8M-urea. Ligandin has been shown previously to contain substantial amounts of ordered structure in the presence of 8M-urea (Listowsky et al., 1976) and perhaps the fourth thiol group is situated within the stable region of the protein. N-(4-Dimethyl-amino-3,5-dinitrophenyl)maleimide also reacts with at least three thiol groups; it is possible that it also reacts with the fourth, but since p-mercuribenzoate was the only means of measuring unchanged thiol groups this could not be assessed.

The reaction of two cysteines with N-ethylmale-imide did not result in the loss of enzymic activity. When two cysteines were modified with the bulkier 5,5'-dithiobis-(2-nitrobenzoic acid), however, a small loss of activity was observed. The maximum loss of activity required the modification of three cysteines with p-mercuribenzoate or N-(4-dimethylamino-3,5-dinitrophenyl)maleimide implying that the reaction with one cysteine in particular affected the enzymic and binding activities. This is borne out by the titra-tion with p-mercuribenzoate at pH 8.2 where there is no loss of activity until the titration of the third thiol group.

It therefore appears that one thiol in particular is involved in the active site of ligandin. The active site consists of two adjacent binding sites, one of which is hydrophobic and a second which binds glutathione. The proximity of these sites allows glutathione to react with hydrophobic substrates which are electro-philic giving ligandin its enzymic activity. The site for the binding of ligands and substrates is thought to be common (Jakoby et al., 1976) and this is supported by the present observation that the thiol

reagents affected the binding of ligands in the same manner as the enzymic activity. Only the binding affinity was affected, however, 1 mol of oestrone sulphate or bilirubin binding per mol of ligandin despite the modification. This may reflect the size of the binding site, the site still being able to accommo-date ligands although less effectively even with part of the site blocked. This could explain why p-mercuribenzoate had a greater effect on the binding of bilirubin than on the smaller oestrone sulphate. It may also explain why sulphobromophthalein was able to prevent reaction with p-mercuribenzoate, whereas oestrone sulphate was not, although the protection with sulphobromophthalein may have been due to its higher association constant (Tipping et al., 1976c) and concentration as well as its larger size. The effect of both p-mercuribenzoate and N-(4-dimethylamino-3,5-dinitrophenyl)maleimide on the circular-dichroism and visible difference spectra of bilirubin bound to ligandin suggests that the modification does alter the conformation of this ligand bound to ligandin.

As the binding site for glutathione is also involved in catalysis the effects of the modification could be explained by reaction in this site. However, the affinity of glutathione was only slightly decreased after the modification with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide so the thiol group is not likely to be associated with glutathione binding.

To summarize, a thiol group appears to be as-sociated with the hydrophobic binding site of ligandin. When modified, the affinities for ligands and the catalytic activities of ligandin are decreased significantly.

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