Biochem. J. (1978) **175**, 47–51
Printed in Great Britain

Polypeptide Linkages and Resulting Structural Features as Powerful Chromogenic Factors in the Lowry Phenol Reaction

STUDIES ON A GLYCOPROTEIN CONTAINING NO LOWRY PHENOL-REACTIVE AMINO ACIDS AND ON ITS DESIALYLATED AND DEGLYCOSYLATED PRODUCTS

By ALBERT M. WU,*† JUNE C. WU‡ and ANTHONY HERP*
**Department of Biochemistry, New York Medical College, Valhalla, NY 10595, and ‡Chemistry Laboratory, Westchester Medical Center, Valhalla, NY 10595, U.S.A.*

(*Received* 25 *January* 1978)

With bovine serum albumin as the reference standard, the armadillo salivary-gland glycoprotein, although containing no chromogenic amino acids and only small amounts of colour-yielding peptides [Chou & Goldstein (1960) *Biochem. J.* **75**, 100–115], is highly reactive in the Lowry phenol protein assay [Wu & Pigman (1977) *Biochem. J.* **161**, 37–47]. After desialylation and Smith degradation of the glycoprotein, the Lowry phenol value increased by 13 and 30% respectively, which suggests that both sialic acid and *N*-acetylhexosamine exert shielding effects in this reaction. Acid hydrolysis for 30min decreased the Lowry phenol value by more than 45%, which indicates that the peptide linkages and steric features affect the Lowry phenol reactivity. After hydrolysis for up to 6h, the remaining Lowry phenol value of the partially hydrolysed core protein paralleled the amount of unhydrolysed peptides, inferring that both acid-sensitive and acid-resistant chromophoric peptides are fairly evenly distributed along the whole polypeptide chain. As with bovine serum albumin, more than 80% of the colour yield obtained in the Lowry phenol assay with this glycoprotein is Cu^{2+}-dependent.

Because of its simplicity, rapidity and high sensitivity, the phenol method of Lowry *et al.* (1951) has been widely used for the determination of proteins, including glycoproteins. The colour yield of this reaction is considered to arise mainly from tyrosine, phenylalanine and tryptophan, and to some extent from the sequence of certain amino acids bearing functional side groups, such as arginine, glutamic acid and histidine (Chou & Goldstein, 1960). Although the protein core of the armadillo salivary glycoprotein contains none of the aforementioned chromogenic amino acids, and only a few chromogenic peptides, it shows an unusually high reactivity in this reaction (Wu & Pigman, 1977). Consequently, other chromogenic factors, such as the nature of the peptide-bonded amino acids or steric features of the glycoprotein, seem to be responsible for its strong reactivity in the Lowry phenol reaction.

The purpose of the present paper is to investigate the effect of desialylation, deglycosylation and timed acid hydrolysis of this glycoprotein on the values obtained in the Lowry phenol assay and to delineate the chromogenic factors responsible for the effects observed.

† Present address: Department of Microbiology, Columbia Medical Center, 701 West 168th Street, New York, NY 10032, U.S.A.

Materials and Methods

Bovine serum albumin was obtained from Mann Research Laboratories, Rutherford, NJ, U.S.A. Folin–Ciocalteu reagent, from Fisher Scientific Co., Fairlawn, NJ, U.S.A., was diluted 1:1 (v/v) with water. The alkaline $CuSO_4$ solution was prepared freshly by adding 0.5ml each of 2% (w/v) potassium sodium tatrate and 1% $CuSO_4$, $5H_2O$ to 50ml of 2% Na_2CO_3 in 0.1M-NaOH.

Armadillo submandibular glycoprotein, fraction A, was prepared by the method of Wu & Pigman (1977). For desialylation, a sample of the glycoprotein, dissolved in 0.01M-HCl, was hydrolysed at 80°C for 40min and dialysed at 4°C against 20 vol. of water for 48h, with frequent changes of water. The non-diffusible material was collected, freeze-dried, and is designated desialylated armadillo submandibular glycoprotein.

Deglycosylation of the armadillo submandibular glycoprotein was achieved by Smith degradation (Goldstein *et al.*, 1965). The desialylated glycoprotein was dissolved in water to which an equal volume of 0.12M-sodium periodate was added. Oxidation was performed at 0°C in an ice bath in the dark. When the uptake of oxidant was essentially complete, the reaction was stopped by the addition of an excess of ethylene glycol. The solution was dialysed against

water and the non-diffusible product reduced with $NaBH_4$ (1 mg/mg of desialylated glycoprotein) at 24°C overnight. To the reaction mixture, HCl was added to make the solution 0.5 M. After 24 h at room temperature, the solution was exhaustively dialysed against water and the non-diffusible fraction freeze-dried and is called deglycosylated glycoprotein.

Analytical procedures

The Lowry phenol value was measured by the procedure of Lowry *et al.* (1951) and is always expressed with crystalline bovine serum albumin as the reference standard. To 0.5 ml of protein sample in a 13 mm × 150 mm test tube, 2.5 ml of alkaline $CuSO_4$ solution was added, and mixed. The mixture was left at room temperature for 20 min, and then 0.25 ml of the phenol reagent was added, mixed, and the A_{750} read after a further 30 min. For the Lowry phenol determination in the absence of Cu^{2+} ion, an equal volume of water was substituted for the copper reagent. Sialic acid was determined by the resorcinol method (Svennerholm, 1957), with *N*-acetylneuraminic acid as the standard. Total hexosamine was analysed, as described by Boas (1953), after hydrolysis of the sample in 6 M-HCl for 4 h at 104°C. For the determination of fucose, the cysteine/H_2SO_4 reaction of Dische & Shettles (1948) was used, with a heating period of 10 min. Galactose was determined as described by Dische & Danilchenko (1967).

Mild acid hydrolysis for desialylation. Samples were dissolved in 0.01 M-HCl and hydrolysed at 80°C for 3 h, cooled and evaporated to dryness under vacuum; the residue was dissolved in water for the protein assay.

Amino acid analysis and measurement of Lowry phenol protein value as a function of acid hydrolysis time. Conc. HCl (2 ml) was added to an equal volume of an aqueous 1 mg/ml sample in a screw-cap Pyrex tube. The solutions were hydrolysed at 110°C for 30 min to 22 h, cooled and evaporated to dryness under vacuum. For amino acid analysis, the residues were dissolved in 10 ml of 0.2 M-sodium citrate buffer, pH 2.2, filtered, and processed with a Beckman model 120B amino acid analyser, under the conditions of Spackman *et al.* (1958). For Lowry protein determination, the residues were taken up in 10 ml of water and insoluble particles removed by centrifugation at 10000*g* for 20 min.

Results

The carbohydrate moiety of armadillo submandibular glycoprotein consists mainly of *N*-acetylgalactosamine (26.6 g%) and *N*-acetylneuraminic acid (13.3 g%). Six amino acids constitute 98 mol% of the protein core: serine and threonine (40–45% of the molar amino acid composition), alanine, glutamic acid, glycine and valine. Neither chromogenic amino acids nor proline and methionine were detected. The chemical composition of the glycoprotein before and after mild acid hydrolysis, and after Smith degradation, is given in Table 1. Mild acid treatment (pH 2 at 80°C) for 30 min released almost all of the sialic acid, whereas the *N*-acetylgalactosamine and amino acid contents remained unchanged. Smith degradation of the desialylated product removed more than 96% of the amino sugar, but the amino acid composition of the deglycosylated material was closely similar to that of the native one.

Evaluation of protein content

The protein value was determined by the method of Lowry *et al.* (1951), in the presence and absence of Cu^{2+}, and by automated amino acid analysis. In the intact armadillo submandibular glycoprotein, the value calculated by amino acid analysis was 40%

Table 1. *Relative molar composition of intact, desialylated, deglycosylated and unhydrolysed peptides after 3 h of hydrolysis of armadillo submandibular-gland glycoprotein*

Samples contained less than 1% of arginine, aspartic acid, isoleucine, leucine, lysine and fucose, and none or traces of cysteine, histidine, methionine, phenylalanine, proline, tryptophan, tyrosine and galactose. Values for unhydrolysed peptides were determined by subtractive amino acid analysis (see Table 2). Abbreviation: N.D., not determined.

	Glycoprotein composition (mol/100 mol of amino acids)			
Component	Intact	Desialylated	Deglycosylated	After 3 h acid hydrolysis
N-Acetylneuraminic acid	7.5	0.4	0	N.D.
Hexosamine	23.0	22.5	1.5	N.D.
Threonine	35.5	35.9	36.2	32.3
Serine	19.6	20.7	20.4	12.2
Glutamic acid	9.9	9.9	9.6	12.2
Glycine	14.4	13.0	12.8	9.0
Alanine	6.0	6.0	6.6	2.0
Valine	13.2	13.1	12.9	32.3

Table 2. *Comparison of protein values in intact, desialylated and deglycosylated armadillo submandibular-gland glycoprotein as determined by the Lowry method, the Lowry method without Cu^{2+} ion and amino acid analyser*

Bovine serum albumin was used as reference standard. The sum of total anhydroamino acids is expressed in g/100g of dried sample. Carbohydrate content is expressed as the sum of *N*-acetylneuraminic acid and *N*-acetylhexosamine. Each value is the mean of four separate analyses ± S.D. Abbreviations: AAA, protein from amino acid analyser; LP, protein determined by Lowry *et al.* (1951) method; LP(−Cu^{2+}), protein determined by Lowry method without Cu^{2+}.

	Glycoprotein composition (g/100g dry wt.)				LP/AAA	LP(−Cu^{2+})/LP (%)
Glycoprotein	LP	LP(−Cu^{2+})	AAA	Carbohydrate		
Native	87.2±4.0	13.0±1.2	53.5±0.6	40.7±1.2	1.63	85
Desialylated	106.6±3.9	14.0±0.8	60.9±0.5	32.0±0.9	1.75	87
Deglycosylated	172.0±4.2	36.0±0.5	90.2±0.7	3.0±0.3	1.90	79

Table 3. *Lowry phenol reactivity and unhydrolysed peptide content of armadillo submandibular-gland glycoprotein after acid hydrolysis*

Hydrolysis was carried out at 110°C in 6M-HCl. Results are expressed as g of unhydrolysed peptide/100g of glycoprotein [(100g of protein −sum in g of hydrolysed protein)/100g of protein]. Values were determined by amino acid analyser. Abbreviations: UHP, unhydrolysed peptide; LP, protein determined by the method of Lowry *et al.* (1951).

Hydrolysis time (h)	UHP (g/100g of glycoprotein)	LP (g/100g of glycoprotein)	UHP/LP	LP reactivity (% difference)*
0.5	75.0	97.8	1.30	−20
1	56.0	74.5	1.33	−18
2	37.9	51.9	1.37	−16
3	29.3	47.8	1.63	0
4	21.7	28.0	1.29	−21
5	11.6	15.8	1.36	−17
6	9.2	12.5	1.36	−17

* Compared with LP reactivity of native glycoprotein.

lower than the value obtained by the Lowry phenol procedure, with bovine serum albumin as the reference standard. When the glycoprotein was treated by mild acid hydrolysis under the conditions found suitable for the removal of sialic acid (pH2 at 80°C), the Lowry protein value increased gradually with time of hydrolysis by 13 and 15%, after 60 and 90min respectively. The desialylated material, subjected to Smith degradation, whereby 96% of the hexosamine was destroyed, displayed a 30% increase in reactivity, compared with that found for the native glycoprotein. These results indicate that probably both sialic acid and hexosamine exhibit shielding effects in the Lowry phenol reaction. More than 80% of the colour yield in this reaction is Cu^{2+}-dependent (Table 2); the actual mechanism of the remaining 20% Cu^{2+}-independent colour development is unexplained.

(A) *Change in Lowry phenol value of the intact glycoprotein as a function of hydrolysis time.* The condition for protein hydrolysis used was 6M-HCl at 110°C. To compensate for the colour arising from humin formation during acid hydrolysis of the glycoprotein, a mixture of similarly hydrolysed carbohydrates (0.26mg of *N*-acetylgalactosamine/ml and 0.13mg of *N*-acetylneuraminic acid/ml, equivalent to the amount of these amino sugars in 1mg of glycoprotein/ml) was used as blank. The colour produced by this mixture varied between 7 and 15%, with bovine serum albumin as standard, and all values were corrected for. The proportion of unhydrolysed material remaining after timed hydrolysis, shown in Table 3, was calculated by subtraction of the 22h-hydrolysis values. In the latter, correction for destruction of serine and threonine and for unhydrolysed valine was applied (Wu & Pigman, 1977). After acid hydrolysis for up to 6h, the remaining Lowry phenol reactivity of the partially hydrolysed core protein paralleled the amount of unhydrolysed peptides, as shown by the relatively constant ratio of the Lowry protein values to unhydrolysed peptides (Table 3). These unhydrolysed peptides exhibited over 80% of their original reactivity. When the glycoprotein was hydrolysed for 22h, about 20% of the valine was still in peptide form, and the Lowry value was of the order of 10% of the original value, corresponding to 17% of that of bovine serum albumin.

(B) *Comparison of Lowry phenol reactivity, after graded acid hydrolysis, between bovine serum albumin*

and deglycosylated glycoprotein. To avoid any interference in the Lowry phenol reaction by the oligosaccharide chains, the armadillo submandibular glycoprotein was deglycosylated. The Lowry reactivity of the deglycosylated compound, containing only 3% *N*-acetylhexosamine, was 1.73 times that of bovine serum albumin. After 30min of hydrolysis, however, more than 45% of its Lowry phenol reactivity was destroyed, whereas under the same conditions less than 2% of the value obtained in the Lowry phenol assay was lost by bovine albumin. This drastic change in Lowry value is thought to be due to a loss of acid-sensitive peptides and perhaps to a change in steric features. The hydrolysates of both proteins gave a similar decrease in the rate of reactivity between 30min and 2h, indicating that the reactivity of the glycoprotein is similar to that of the chromogenic amino acids containing serum albumin. After hydrolysis for 3 and 4h, the response of the unhydrolysed peptides of the glycoprotein closely approximated that of the native product. The molar composition of the unhydrolysed peptide, after 3h of acid hydrolysis, is shown in Table 1. Five amino acids, glutamic acid, glycine, serine, threonine and valine, make up 98% of its composition, two-thirds being threonine and valine, so that it seems that peptides containing these two amino acids play an important role in this reaction.

After 22h of hydrolysis, the Lowry phenol value for bovine serum albumin was 35% and that for the deglycosylated glycoprotein 17% of their respective starting value. Since the glycoprotein contains no detectable chromogenic amino acid, it is assumed that the remaining 20% of peptide-linked valine (Wu & Pigman, 1977) is responsible for the colour yield, whereas for albumin the residual reactivity is attributed to the presence of chromogenic amino acids (Chou & Goldstein, 1960).

Discussion

The value of the protein content of the armadillo submandibular glycoprotein is much lower by amino acid analysis than by the Lowry phenol method. Thus the total recovery, in terms of gross composition, was 129.9% when protein was measured by the Lowry phenol method, and was 30% higher than when protein was determined by amino acid analyser (Table 2). These results indicate that this glycoprotein shows an unusually high reactivity in the Lowry phenol reaction.

The Lowry phenol method yields a combination of colours arising from the reduction of phosphomolybdate or phosphotungstate salts in the Folin–Ciocalteu reagent by such chromophoric amino acids as tyrosine, phenylalanine and tryptophan, and by the biuret reaction, which is based on the co-ordination complex of peptide bonds with the alkaline copper in the reagent (Chou & Goldstein, 1960). On the basis of amino acid composition of this salivary glycoprotein (Table 1), it seems that the biuret reaction is the main factor in the Lowry *et al.* (1951) protein assay. Since its protein core contains only weakly chromogenic peptides composed of glutamic acid, alanine and glycine, some other factors, such as other chromogenic peptides, oligosaccharide side chains, or structural peculiarities, are involved in the chromophore. In the present study, we have investigated this aspect and have found that both the desialylated and deglycosylated protein increase in reactivity, by 15 and 30% respectively. These results indicate that both sugar components may exhibit shielding effects in the Lowry reaction. Such an effect has also been observed by Boursnell *et al.* (1970) and we have obtained similar results with ox and sheep submaxillary glycoproteins (A. M. Wu, unpublished work).

Isolation of the unhydrolysed peptides from the glycoprotein would be a logical step to identify the main chromogenic peptides in this glycoprotein, but because of the paucity of material available, this was not possible. However, subtractive amino acid analysis provided some information about possible chromogenic peptides in this glycoprotein; as a result of acid hydrolysis (6M-HCl at 110°C), especially after 30min, the reactivity decreased by more than 45%. This is very convincing evidence that the peptide linkages of this glycoprotein and steric features thereof play an essential role in the Lowry phenol reactivity. After hydrolysis for up to 6h, the parallelism between the remaining Lowry reactivity and the amount of unhydrolysed peptides showed that both the acid-sensitive and acid-resistant chromogenic peptides are fairly evenly distributed along the entire polypeptide chain.

Since proline and cysteine are important in determining the secondary and tertiary structure of proteins, their absence from the armadillo glycoprotein infers a linear or semi-rigid rod-like structure in either a low- or high-ionic-strength environment. Such structures increase the opportunities for peptide chains to react with copper and phenol reagent. Sulphated glycoproteins from dog gastric mucosa, which contain a considerable amount of proline, show low reactivity (*Pamer et al.*, 1968) in the Lowry *et al.* (1951) assay; consequently, the negligible amount of proline in this glycoprotein might be an essential facet for its high reactivity in the Lowry assay.

As with bovine serum albumin (Chou & Goldstein, 1960), more than 80% of the colour yield exhibited by the armadillo glycoprotein in the Lowry phenol assay is Cu^{2+}- dependent (Table 2). We are not sure whether the colour value of the residual 20% of chromophoric peptide is Cu^{2+}- dependent and colour

development is due to contaminating Cu^{2+} ions in the reagents or is the result of a tyrosine-type reduction of the phosphotungstate and phosphomolybdate salts in the phenol reagent.

This work was supported by a grant from the National Institute of Dental Research (DE 04084-04).

References

Boas, N. P. (1953) *J. Biol. Chem.* **204**, 553–563
Boursnell, J. C., Hartree, E. F. & Briggs, P. A. (1970) *Biochem. J.* **117**, 981–988
Chou, S. & Goldstein, A. (1960) *Biochem. J.* **75**, 109–115
Dische, Z. & Danilchenko, A. (1967) *Anal. Biochem.* **21**, 119–124
Dische, Z. & Shettles, L. B. (1948) *J. Biol. Chem.* **175**, 595–603
Goldstein, I. J., Hay, G. W., Lewis, B. A. & Smith, F. (1965) *Methods Carbohydr. Chem.* **5**, 361–370
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Pamer, T., George, B., Glass, J. & Horowitz, M. I. (1968) *Biochemistry* **7**, 3821–3829
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
Svennerholm, L. (1957) *Biochim. Biophys. Acta* **24**, 604–611
Wu, A. & Pigman, W. (1977) *Biochem. J.* **161**, 37–47