The Electrophoretic Mobilities of 5-Dimethylaminonaphthalene-1-sulphonyl-Glycopeptides and their Relation to Molecular Weight

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The relationship between the electrophoretic mobility at pH 2.1 of dansyl-glycopeptides of known composition and their molecular weight is shown to conform with a model equation previously derived for peptides. A dansyl-glycopeptide prepared from hen's-egg ovotransferrin is degraded sequentially with two glycosidases. The molecular weight of each glycopeptide intermediate formed is determined from its electrophoretic mobility. From successive molecular-weight changes, the number and type of sugar residues lost from the parent glycopeptide can be decided and the probable composition of each intermediate determined. The notion that the method has considerable application and would permit analysis of very small quantities of glycopeptides is discussed.

In structural studies of biological polymers, where large numbers of low-molecular-weight derivatives are often generated and must afterwards be characterized, simple methods of molecular-weight determination are invaluable. A method introduced by Offord (1966) relating the electrophoretic mobility of peptides to their charge and molecular weight has been widely used in protein studies. The object of the present work was to modify this method so that the molecular weights of glycopeptides might also be conveniently determined. This achieved, it was then found that by coupling the modified procedure with systematic enzymic degradation of the oligosaccharide moiety of a glycopeptide certain structural detail could be deduced. Some of the difficulties normally encountered in such structural studies, for example the requirement for comparatively large amounts of glycopeptide, the inadequacies of chromatographic methods and 'fingerprint' techniques (Moczar & Moczar, 1967) and the relative insensitivity of carbohydrate stains, were avoided.

Experimental

Standard conditions for the measurement of electrophoretic mobilities relative to dansyl-aspartic acid

Dansyl-glycopeptide samples were electrophoresed together with samples of Dns-Asp on Whatman 3MM paper for 50 min at 3 kV (80 V/cm) with a volatile aqueous buffer at pH 2.1 (Ambler, 1963). The temperature was approx. 30°C. Dns-OH behaved as a neutral compound like glucose under these electrophoretic conditions and provided a

convenient measure of electroendosmosis (Wenn, 1969; Bailey & Ramshaw, 1973). Mobilities were determined from measurements of the distances between the Dns-OH marker (mid-point of spot) and the leading edges of the dansyl-glycopeptide and Dns-Asp bands.

Preparation of dansyl-2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose and dansyl-aspartic acid

Synthetic 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose (Asn-GlcNAc) was a gift from Dr. T. H. Plummer, Jr. The preparation (2 mg) was dissolved in 1.0 ml of water and 2 mg of dansyl chloride in 1.0 ml of acetone added. After 3 h at approx. 20°C in the dark the mixture was electrophoresed at pH 2.1. Only a single fluorescent band was observed besides that of Dns-OH. The band was cut out and eluted from the paper with 1.0% (v/v) aq. NH₃. A sample of the derivative, Dns-Asn-GlcNAc, was re-electrophoresed under standard conditions and its mobility relative to Dns-Asp determined. Dns-Asp was prepared from L-aspartic acid by reaction with dansyl chloride in a similar way. It was recovered pure by electrophoresis at pH 2.1.

Preparation of a dansyl-glycopeptide from hen's-egg ovotransferrin

A basic glycopeptide fraction was prepared from hen's-egg ovotransferrin by digestion with pepsin and subtilisin according to the method of Williams (1968). It was composed of approx. 75% total hexose by wt. and possessed the amino acid composition (mol. of residue/mol. of peptide) Asx (1.0), Arg (1.0),

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Table 1. *Composition of a glycopeptide from ovotransferrin*

The molar composition of three individual preparations of ovotransferrin glycopeptide after carboxypeptidase A treatment (see the text) is given. Asx was determined as Asp. Sugars determined by g.l.c. were silylated (Sweeley *et al.*, 1963). —, Not determined.

Amino acid or monosaccharide residue	Means of determination	Molar ratio of residue relative to Asx		
		Prepn. 1	Prepn. 2	Prepn. 3
Asx	Amino acid analyser	1.0	1.0	1.0
Arg	Amino acid analyser	1.0	1.0	1.0
Total hexose	Orcinol method of Winzler (1958)	3.0	3.0	3.2
Total hexosamine	Method of Rondle & Morgan (1955)	4.8	4.8	5.0
Man	G.l.c.	2.7	2.7	3.0
GlcNAc	G.l.c.	—	4.8	—

Thr (about 0.8). The amino acid analysis was carried out with a Technicon amino acid analyser after 24h hydrolysis of the peptide with 5.7M-HCl at 105°C. Prolonged electrophoresis for 2.5h at pH 6.5 revealed two glycopeptide components. The component of greater mobility only contained the amino acids Asx (1.0) and Arg (1.0). A homogeneous preparation was prepared by treatment with carboxypeptidase A to remove all C-terminal threonine. It migrated as a single band under all electrophoretic conditions and gave a single, symmetrical peak in sedimentation studies in the ultracentrifuge. Three such preparations were analysed by the methods summarized in Table 1. The molecular weight was determined by exclusion chromatography on Sephadex G-25 and by the method of equilibrium centrifugation (Yphantis, 1964).

The same preparation (20mg) in 1 ml of aq. 0.1% NaHCO₃ was treated with carboxypeptidase B to release C-terminal arginine and yield a glycopeptide containing only aspartic acid (or asparagine). After 24h incubation at 37°C, when the concentration of free arginine detected in samples of the digest electrophoresed at pH 2.1 was maximal, 6mg of dansyl chloride in 1.5 ml of acetone was added and the mixture left for 3h at approx. 20°C in the dark. It was then desalted on Sephadex G-25 and electrophoresed at pH 2.1. Of the two major fluorescent bands observed on the electrophoretogram besides Dns-OH, only one reacted with aniline-diphenylamine carbohydrate stain (Schwinn & Bevenue, 1956). This band was cut out and eluted with aq. 1.0% (v/v) NH₃. The other, identified as Dns-Arg, was discarded. The eluate was freeze-dried to yield 12mg of dansyl-glycopeptide. A small sample was re-electrophoresed at pH 2.1 under standard conditions to determine its mobility relative to Dns-Asp.

Preparation of an α -mannosidase from bull epididymis

The epididymes dissected from two testes were homogenized and extracted in 1.5 litres of 0.05M-acetic acid at 0°C. After centrifugation the super-

natant was made 5mM in zinc acetate and cold saturated (NH₄)₂SO₄ was added. Protein precipitated at 0°C, as the saturation of (NH₄)₂SO₄ in the extract increased from 20% to 50%, was re-dissolved in 50ml of 0.05M-sodium citrate buffer made 10mM in zinc acetate and adjusted to pH 4.5 with 1M-NaOH. A 20–40% (v/v) acetone cut was made at 0°C and the precipitate taken up in 30ml of 0.025M-citrate buffer, pH 4.5, 5mM in zinc acetate. This solution was dialysed against 2.5mM-zinc acetate at 0°C and the dialysis residue freeze-dried. The specific activity of the α -mannosidase in 0.05M-citrate buffer, pH 4.4 at 37°C, was approx. 0.3 μ mol of *p*-nitrophenol liberated from *p*-nitrophenyl D- α -mannoside/min per mg of dry residue. Residual *N*-acetyl- β -glucosaminidase activity was blocked with 2-acetamido-2-deoxygluconolactone. Glucosaminic acid was prepared from D-glucosamine by the method of Hope & Kent (1955) and acetylated as described by Findlay *et al.* (1958).

Preparation of N-acetyl- β -glucosaminidase from ram testes

An acetic acid extract of ram testes (Bengers Ltd., Holmes Chapel, Cheshire, U.K.) was fractionated with (NH₄)₂SO₄ (Borooah *et al.*, 1961). The precipitate from the 30–40% cut was re-dissolved in water, dialysed, and the dialysis residue stored frozen at –12°C. The specific activity of *N*-acetyl- β -glucosaminidase in the crude precipitate dissolved in 0.05M-citrate buffer, pH 4.4 at 37°C, was 0.88 μ mol of *p*-nitrophenol liberated from *p*-nitrophenyl *N*-acetyl- β -D-glucosamine/min per mg wet wt. No α -mannosidase or β -galactosidase activity could be detected in the preparation (cf. Caygill & Jevons, 1964).

Treatment of dansyl-glycopeptide with specific glycosidase

Duplicate samples (0.05–1.0mg) of dansyl-glycopeptide (or products of its enzymic degradation) were dissolved in 0.5ml of 0.05M-citrate buffer adjusted to

Table 2. Summary of data obtained for glycopeptide derivatives generated during the sequential enzymic degradation of a dansyl-glycopeptide prepared from ovotransferrin

For details of the enzymic digestion and method of mobility determination see text. The approximate molecular weights of the five derivatives observed were determined from their respective mobilities by using a logarithmic plot (see Fig. 1). The molecular weight and composition of the parent glycopeptide were determined by gross analysis (see the text and Table 1), and the composition of derivative 1 was confirmed by similar means. Derivative 5 co-electrophoresed with a synthetic dansyl-glycopeptide (b, Fig. 1) possessing the same structure as that deduced. Derivatives 2 and 3 appeared as transients (see the text) during digestion with α -mannosidase. The composition of each derivative is deduced from each successive molecular-weight change and knowledge of which glycosidase effected the change.

Derivative	Mobility relative to Dns-Asp	Approx. mol.wt. (from log plot)	Approx. mol.wt. change	Glycosidase effecting change	Type (and no.) of residues lost	Composition deduced	Mol.wt. from composition
Parent glycopeptide	0.41	—				Dns-Asn(Man ₃ , GlcNAc ₂)	1866 (standard)
1	0.51	1250	616	N-Acetyl- β -glucosaminidase	GlcNAc (3)	Dns-Asn(Man ₃ , GlcNAc ₂)	1257
2	0.55	1085	165	α -Mannosidase	Man (1)	Dns-Asn(Man ₂ , GlcNAc ₂)	1095
3	0.60	925	160	α -Mannosidase	Man (1)	Dns-Asn(Man ₁ , GlcNAc ₂)	933
4	0.66	775	150	α -Mannosidase	Man (1)	Dns-Asn(GlcNAc ₂)	771
5	0.79	570	205	N-Acetyl- β -glucosaminidase	GlcNAc (1)	Dns-Asn(GlcNAc ₁)	566

pH 4.5. One sample was treated with α -mannosidase, the other with *N*-acetyl- β -glucosaminidase. The total glycosidase activity of each was approx. $0.3 \mu\text{mol}$ of *p*-nitrophenol liberated/min, but it was not critical. The mixtures were incubated for 24 h at 37°C . During the incubation small volumes of the medium ($50 \mu\text{l}$) were periodically removed and electrophoresed at pH 2.1 under standard conditions. In this way the course of any enzymic degradation that had occurred could be followed. The mobility of any transient fluorescent intermediate formed could be determined. When digestion was complete and the glycopeptide maximally degraded by whichever of the two glycosidases had proved active, only a single fluorescent band of product was seen on electrophoresis. After determination of the mobility under standard conditions, this band was cut out, eluted and freeze-dried. The dried, degraded product was then re-dissolved in buffer and redigested as above. In this sequential fashion dansyl-glycopeptide was broken down until only a single sugar residue remained; the dansyl-glycopeptide derivatives observed as intermediates are listed in Table 2.

Results and Discussion

From the data given in Table 1 the composition of the ovotransferrin glycopeptide after carboxypeptidase A treatment is $\text{Asx}(\text{Man}_3, \text{GlcNAc}_5)\text{Arg}$. It is most probable that the aspartyl type of residue is in fact an aspartamido group (Williams, 1968), an assumption substantiated in the present work (Table 2). The value of the theoretical molecular weight of $\text{Asn}(\text{Man}_3, \text{GlcNAc}_5)\text{Arg}$, 1804, is in good agreement with the experimental values 1640 ± 100 and 1800 ± 300 (mean $\pm$ S.E.M. for three observations) determined by chromatography and equilibrium centrifugation respectively. The glycopeptide derived from $\text{Asn}(\text{Man}_3, \text{GlcNAc}_5)\text{Arg}$ by removal of *C*-terminal arginine must thus have a molecular weight of 1633 and its dansyl derivative a molecular weight of 1866.

Offord (1966) demonstrated that the electrophoretic mobility of peptides at pH 6.5 relative to aspartic acid was a function of their charge and molecular mass. Of two possible model equations derived to explain the relationship only one agreed with experimental data:

$$m = k' \varepsilon M^{-2/3}$$

where m was the mobility relative to aspartic acid, k' a constant, ε the net electrostatic charge and M the mass of the molecule. A logarithmic plot of peptide mobility versus molecular weight was shown to have a slope of almost exactly $-2/3$.

In the present work, three dansyl compounds are of known molecular weight and mobility, since they have either been synthesized or characterized (a fourth was subsequently characterized; see below). They are (a) Dns-Asp (mol.wt. 365, $m=1.0$), (b) Dns-

Fig. 1. Logarithmic plot of electrophoretic mobility of dansyl-glycopeptides at pH 2.1 versus molecular weight

Δ (a, b, c), Three standard dansyl compounds of known molecular weight and mobility (see the text) were used to construct the plot. $\bullet$ (1, 2, 3, 4, 5), Mobilities of dansyl-glycopeptide derivatives (see Table 2). All mobilities are relative to Dns-Asp.

Asn-GlcNAc (mol.wt. 569, $m=0.79$) and (c) Dns- $\text{Asn}(\text{Man}_3, \text{GlcNAc}_5)$ (mol.wt. 1866, $m=0.41$). The mobility and molecular-weight values of these compounds are plotted logarithmically in Fig. 1. The slope of the straight line through the points is -0.6 , close to the theoretical value predicted from Offord's (1966) model equation.

By using the logarithmic plot, molecular weights can be determined from mobility values for the family of five dansyl-glycopeptides derived sequentially from Dns- $\text{Asn}(\text{Man}_3, \text{GlcNAc}_5)$ by enzymic degradation (Table 2). Knowledge of successive molecular weight changes and of the specific glycosidase effecting any given change immediately provides the type and number of sugar residues lost from the parent dansyl-glycopeptide. Thus Dns- $\text{Asn}(\text{Man}_3, \text{GlcNAc}_5)$ can be degraded to Dns- Asn-GlcNAc by α -mannosidase and *N*-acetyl- β -glucosaminidase in the sequence shown (Table 2). Since glycosidases are exoenzymes the order in which successive monosaccharide residues become susceptible to them puts certain restraints on the possible linear sequence of the oligosaccharide moiety of the glycopeptide (Wenn, 1969).

It is surprising that no transient glycopeptides were observed during the degradation of Dns- $\text{Asn}(\text{Man}_3,$

GlcNAc₅) to Dns-Asn (Man₃, GlcNAc₂) with *N*-acetyl- β -glucosaminidase. Partial acid hydrolysis of Dns-Asn(Man₃, GlcNAc₅) (Wenn, 1969) gave a fluorescent derivative that had the mobility (0.44) expected for Dns-Asn(Man₃, GlcNAc₄). A possible explanation is that the Dns-Asn(Man₃, GlcNAc₅) molecule, once associated with the enzyme, is degraded completely to Dns-Asn(Man₃, GlcNAc₂) before release. The three terminal *N*-acetyl- β -D-glucosamine molecules might feed sequentially through the active site, being hydrolysed one immediately after the other. Because of the absence of the intermediates the composition, Dns-Asn(Man₃, GlcNAc₂), assigned to derivative 1 (Table 2) was confirmed by gross analysis by using the methods listed in Table 1. The theoretical molecular weight of this derivative is 1257, very close to that of 1250 determined from the logarithmic plot. In fact in no case is the discrepancy between the approximate molecular weight measured from the logarithmic plot and the theoretical molecular weight derived from the composition assigned to each derivative very large (Table 2).

There has been some discussion (Offord, 1966; Bailey & Ramshaw, 1973) concerning the fit of experimental results to the model equation relating mobility and molecular mass. Offord (1966) cautioned against expecting too close an agreement, since molecular weight is possibly a poor measure of the effective mass of a molecule, and factors of molecular shape, which influence mobility, have anyway been ignored. Despite these inconsistencies there is now fairly good agreement between theory and experiment for peptides electrophoresed at either pH 6.5 or pH 1.9 (Bailey & Ramshaw, 1973). The present results are no exception, and indeed dansyl-glycopeptides of the type examined may provide better models for further studies of the mobility-molecular-weight relationship than peptides themselves. Unlike peptides and dansyl-peptides (Gray, 1967), which are polymers composed of a variety of amino acids of quite dissimilar shapes and sizes, a glycopeptide of the type described is principally a polymer consisting of rather uniform monosaccharide residues. Thus on degradation the shape change incurred with changing molecular weight is also comparatively uniform. Further, the amount of charge carried by each dansyl-glycopeptide molecule is exactly the same, since it is solely a function of the dansyl-aspartamido group, common to all. The terminal location of this charge on each dansyl-glycopeptide molecule also provides for uniformity. The charges of peptides, examined at pH 6.5 (Offord, 1966), were carried on the side chains of amino acid residues which could occupy any position, terminal or central, within the peptide.

As a means of determining glycopeptide molecular weight the mobility method should be widely applicable. Oligosaccharides attached to other amino acid residues (e.g. threonine, serine), perhaps through *O*-glycosidic bonds, are, in the present context, essentially no different from an oligosaccharide attached to asparagine via an *N*-glycosidic bond. Glycopeptides containing sugar residues possessing basic or very acidic groups are the exception, but in these cases the offending group might be blocked or removed in order to determine comparable mobility values. Alternatively, it should be possible to tolerate the additional charge and modify the method accordingly, provided suitable compounds are available from which to prepare the logarithmic plot. Once the charged residue is lost from the glycopeptide in the course of degradation, the plot would have to be re-calibrated for the new conditions of charge. If the mobility method can be combined with sequential enzymic degradation it becomes a valuable means of structural analysis limited only by the availability of specific enzymes. The dansyl group as a fluorescent marker gives the method the additional advantage that exceedingly small quantities of glycopeptide, theoretically 0.1–1 nmol (Gray & Hartley, 1963; Gray, 1967), can be examined and detected. The dansyl group is also resistant to acid hydrolysis and dansyl-glycopeptides can be degraded in this way before mobility studies (Wenn, 1969).

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References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
- Bailey, C. J. & Ramshaw, J. A. M. (1973) *Biochem. J.* **135**, 889–891
- Borooh, J., Leaback, D. H. & Walker, P. G. (1961) *Biochem. J.* **78**, 106–110
- Caygill, J. C. & Jevons, F. R. (1964) *Biochem. J.* **90**, 10p
- Findlay, J., Levvy, G. A. & Marsh, C. A. (1958) *Biochem. J.* **69**, 467–476
- Gray, W. H. (1967) *Methods Enzymol.* **11**, 469–475
- Gray, W. H. & Hartley, B. S. (1963) *Biochem. J.* **89**, 59p
- Hope, D. B. & Kent, P. (1955) *J. Chem. Soc. London* 1831–1833
- Moczar, E. & Moczar, M. (1967) *Biochem. J.* **104**, 17p
- Offord, R. E. (1966) *Nature (London)* **211**, 591–593
- Rondle, C. J. M. & Morgan, W. T. J. (1955) *Biochem. J.* **61**, 586–589
- Schwinn, S. & Bevenue, A. (1956) *Science* **123**, 543–544
- Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963) *J. Amer. Chem. Soc.* **85**, 2497–2507
- Wenn, R. V. (1969) Ph.D. Thesis, University of Bristol
- Williams, J. (1968) *Biochem. J.* **108**, 57–67
- Winzler, R. J. (1958) *Methods Biochem. Anal.* **2**, 279–311
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317