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β Elimination and Sulfite Addition as a Means of Localization and Identification of Substituted Seryl and Threonyl Residues in Proteins and Proteoglycans†

D. L. Simpson, Jovan Hranisavljevic,‡ and E. A. Davidson*

ABSTRACT: A reaction is described for qualitative and quantitative determination of substituted hydroxyamino acids in glycoproteins, phosphoproteins, and protein–polysaccharides. The reaction involves β elimination of substituted seryl and threonyl residues and subsequent α–β nucleophilic addition of sulfite ion to the corresponding dehydro analogs resulting in stoichiometric formation of sulfonic acid derivatives, cysteic acid, and 2-amino-3-sulfonylbutyric acid, respectively. The threonyl analog may form more slowly than cysteic acid due to the possibly lower reactivity of the dehydrothreonyl grouping. When this base-catalyzed β elimination is carried out under these conditions in the presence of ^{35}S-labeled sulfite unique localization of substituted loci is ensured. The report describes specific studies with purified protein–polysaccharide from cartilage, casein and phosvitin, which both contain *O*-phosphorylhydroxyamino acids, and bovine submaxillary mucin, a glycoprotein in which both serine and threonine are extensively substituted with carbohydrate. Glucagon, lysozyme, and bovine serum albumin were employed as protein controls, and no cysteic acid was formed under identical reaction conditions and no loss of serine or threonine was observed other than that expected from destruction under the hydrolytic conditions employed. Optical rotatory dispersion studies of sulfite-treated bovine serum albumin or casein indicate a retention of native structure. The sulfite addition products were isolated and characterized by several chemical and chromatographic criteria. Since cysteic acid and 2-amino-3-sulfonylbutyric acid are not resolved on the automatic amino acid analyzer, a quantitative method was developed for their separation and estimation. Dowex 50 chromatography was followed by conversion of the sulfonic acids to their respective trimethsilyl derivatives which were then separated by gas–liquid chromatography. Asparaginyl *N*-glycosidic linkages like those present in ovalbumin, are stable under the reaction conditions as are the hydroxylysyl *O*-glycosides present in collagen. The presence of cysteine, known to give rise to dehydroalanine at high pH, is not critical or limiting, provided that reduction and S-carboxylmethylation of free sulfhydryl or disulfide groups is performed prior to the elimination–addition. Alkali-catalyzed peptide-bond cleavage under the described conditions appears negligible. Experiments with DFP-trypsin suggest that this procedure can be extended to specific labeling of a single serine or threonine residue associated with a catalytic site which, due to its reactivity, has been previously derivatized.

The complete structural elucidation of glycoproteins or proteoglycans entails extensive chemical or enzymatic degradation, isolation of linkage region fragments containing only a single amino acid and characterization of the residual saccharide moieties. In general, although a fair amount of information can be obtained from compositions of glycopeptides after proteolytic digestion, reconstruction of the intact protein structure is incomplete.

The amide nitrogen of asparagine (Marshall and Neuberger, 1954) and the hydroxyl function of serine or threonine

† From the Department of Biological Chemistry, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received December 29, 1971.* This work was supported by Public Health Service Grant AM12074.

‡ Present address: Galenika Research Institute, Zemun, Yugoslavia.
* To whom all correspondence should be addressed.

(Hashimoto and Pigman, 1962; Anderson *et al.*, 1964) are the major covalent loci for attachment of saccharide units to polypeptide chains.

The base-catalyzed β elimination of seryl and threonyl residues has been described for a wide variety of complex proteins (Carubelli *et al.*, 1965; Tanaka *et al.*, 1964; Adams, 1965). Reaction with mild alkali converts these residues to α-aminoacrylic and α-aminocrotonic acid, respectively.

Since the β elimination is complete under optimal conditions, a specific and quantitative reaction of the unsaturated residues would enable one to locate those hydroxylamino acids substituted although a precise assignment of the location of carbohydrate units requires the determination of the complete amino acid sequence of the polypeptide. The reduction of the unsaturated β-elimination products which converts α-aminoacrylic acid to alanine and α-aminocrotonic acid to α-aminobutyric acid, has been reported (Tanaka and Pigman, 1965).

The utilization of sulfite to identify β-eliminated seryl and threonyl residues was first suggested by Harbon (Harbon *et al.*, 1964) who reacted β-eliminated ovine submaxillary mucin with sodium sulfite. However, the yield of cysteic acid was only 55%, the product was not identified and there was no indication of a corresponding product from threonine. In a subsequent study with porcine submaxillary mucin (Harbon *et al.*, 1968), the results indicated that the addition reaction was incomplete, since the overall conversion of the hydroxyamino acids to their sulfite addition products was much less than the theoretical value. In addition, the products were not characterized and it could not be determined if both serine and threonine participated in the β elimination and sulfite addition.

Sulfite addition was also employed in a qualitative fashion to support the presence of dehydroalanine in α-chymotrypsin treated with *p*-toluenesulfonyl chloride and subsequently eliminated with alkali (Strumeyer *et al.*, 1963).

In this paper, we describe conditions under which substituted seryl and threonyl residues in glycoproteins, phosphoproteins, and proteoglycans can be converted to their corresponding sulfonic acid derivatives, cysteic acid and 2-amino-3-sulfonylbutyric acid. The separation and estimation of the Me_3Si derivatives of the sulfonic acid products by gas–liquid chromatography is described. With this method of separation and the use of ^{35}S-labeled sulfite, quantitative and unique localization of substituted seryl and threonyl residues in complex proteins is assured. A preliminary report has been presented (Simpson *et al.*, 1971).

Materials and Methods

Phosvitin and serine *O*-phosphate were products of Calbiochem Corp. Lysozyme and DFP-trypsin were obtained from Worthington Biochemical Corp. α-Casein was a product of the Mann Research Laboratories, Inc. Bovine serum albumin was obtained from Sigma Chemical Co. Sodium bisulfite as the Baker Analyzed reagent was purchased from the J. T. Baker Chemical Co. The [^{35}S]sodium sulfite was obtained from New England Nuclear Corp. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (hereafter simply referred to as "the trifluoroacetamide") was a product of Supelco Inc. of Bellefonte, Pa. All other chemicals used were the best available commercially.

Protein–polysaccharide from porcine costal cartilage was prepared in our laboratory according to a modification of the procedure described by Sajdera and Hascall (1969) using 2 M calcium chloride or 4 M quanidinium chloride as extractant.

Elimination–addition reactions were generally carried out at room temperature at pH 11.5 in the presence of *freshly* prepared 0.1–0.2 M sodium sulfite except for the phosvitin studies which were performed at pH 12.8. The reactions were initially followed by taking aliquots for amino acid analysis at regular intervals to measure hydroxyamino acid loss and sulfonic acid production. Preparative reactions, with or without [^{35}S]sulfite, were carried out after optimal conditions of pH, sulfite ion concentration, and time had been determined for a particular protein.

The time course of reaction was usually 4–24 hr. The pH during reaction was monitored with an Orion Model 801 pH meter and readjusted, if necessary, with alkali. The concentration of protein or polypeptide employed was 1 mg/ml. Reactions were terminated by the addition of sufficient 5 N HCl to adjust the pH to 7.0. After exhaustive dialysis *vs.* water and final desalting on a column (2 × 60 cm) of Sephadex G-15 equilibrated with water, samples were lyophilized and stored in a desiccator for subsequent characterization.

The analysis of all samples for amino acids, glucosamine, and galactosamine was performed on a Beckman Model 120C amino acid analyzer following hydrolysis *in vacuo* in 6 N HCl for 24 or 48 hr. Peak areas on chromatograms were determined using an on-line integrator and compositions were calculated by means of a computer program developed in this laboratory. Data from three standard runs was averaged to provide normalized peak areas. Fresh standards were run every week or whenever fresh ninhydrin was prepared. Appropriate corrections were made for the loss of serine (10%) and threonine (4%) during the hydrolytic procedure.

Recovery of single amino acids from protein hydrolysates was accomplished using the stream-splitting accessory on the amino acid analyzer during analytical or preparative chromatography. In either case, 75% of the sample was diverted to a fraction collector and the remaining was reacted with ninhydrin. [^{14}C]Glycine (15,000 dpm) was routinely included in the sample as a marker.

Optical rotatory dispersion data were obtained using a Cary Model 60 CD spectropolarimeter. Samples were run at a concentration of 1 mg/ml in either 0.01 M Hepes[1]–0.01 M NaCl or 6 M guanidine hydrochloride.

Radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter using an internal standard. Samples were spotted on paper disks and suspended or dissolved in appropriate scintillation mixtures.

Uronic acid was determined by the orcinol (Brown, 1946) or carbazole method (Dische, 1947).

Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

Phosphate was determined by a modification of the method of Fiske–Subbarow (1925) described by Bartlett (1959), after a preliminary digestion of the sample with concentrated sulfuric acid.

The sulfonic acid products were purified from protein hydrolysates by column chromatography, using Bio-Rad Laboratories resin AG-50-W X12 (200–400 mesh) in the hydrogen form. The column was equilibrated with 0.2 M pyridine–acetate buffer (pH 3.0). The sulfonic acid derivatives are not bound to the column under these conditions and can be recovered free of all other amino acids by lyophilization of the effluent. Prior to gas chromatographic studies, samples were

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I: Effect of Alkali Sulfite on Glucagon.[a]

Amino Acid[b]	Lit. Values[c]	Control	24-hr Elimination–Addition
Aspartic	166	170.4	180.3
Threonine	125	125.1	112.9
Serine	166	168.5	165.7
Glutamic	125	124.4	128.8
Glycine	42	42.4	47.8
Alanine	42	43.2	37.8
Valine	42	40.0	39.6
Methionine	42	45.5	46.9
Leucine	83	80.8	78.3
Tyrosine	83	90.0	86.0
Phenylalanine	83	79.7	78.7
Cysteic			2.3

[a] Amino acid analysis of glucagon before and after 24-hr treatment with alkaline sulfite. [b] Values are in residues per 1000 residues; long-column analysis only. [c] Bromer *et al.* (1957).

dried *in vacuo* for 48 hr over NaOH and H_2SO_4 to remove residual pyridine acetate and moisture.

Reduction and S-carboxymethylation, when appropriate, was carried out by a modification of the procedure of Hirs (1967) utilizing 5.0 M guanidine hydrochloride–0.01 M mercaptoethanol at pH 8.5 as solvent.

Gas–liquid chromatographic separation of the sulfonic acid trimethylsilyl derivatives was achieved by a modification of a procedure described by Gehrke (Gehrke *et al.*, 1969). Since 2-amino-3-sulfonylbutyric acid was not available, homocysteic acid was employed as a standard for measurement of retention times since it has the same molecular weight and functional groups. Structures are given in Figure 1. The Me_3Si derivatives were prepared reacting 1 mg of sample with 200 μl of the trifluoroacetamide and 300 μl of acetonitrile, previously dried over anhydrous $CaSO_4$, in a sealed tube with a Teflon cap. The reaction mixtures were heated in an oil bath at 105–107° for 10 min and injected directly onto the columns.

A Perkin-Elmer Model 900 gas chromatography was employed using 6 ft × $^1/_8$ in. columns packed with 1% SE-30 on Gas Chrom Q (80–100 mesh). Samples (0.5–20 μl) were injected with a Hamilton syringe at an injection port temperature of 210°. The temperature was programmed from 80 to 165° at a rate of 10°/min using an attenuator setting of 128X and an amplitude setting of 10X. The flow rate of carrier gas (N_2) was 40 cm^3/min. The conditions for the maximum resolution of the Me_3Si derivatives of cysteic and homocysteic acids by gas–liquid chromatography were determine l experimentally. Sharp symmetrical peaks at 143 and 152°, respectively were obtained, and mixtures were separated and afforded reproducible peaks at the expected temperatures. The Me_3Si derivatives of both compounds have limited stability with that of homocysteic acid being least stable.

Results

Glucagon serves as an ideal control molecule for the elimination–addition reaction since it contains a high percentage

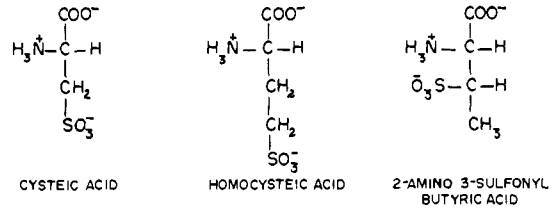

FIGURE 1: Structure of the sulfonic acid products obtained after elimination–addition and hydrolysis. Cysteic acid is derived from substituted seryl residues and 2-amino-3-sulfonylbutyric acid from substituted threonyl residues. Homocysteic acid is not an expected product but was employed as a standard for gas chromotography.

of unsubstituted hydroxyamino acids (14% serine, 10% threonine) and no cysteine. The amino acid analysis of glucagon before and after reaction with alkaline sulfite for 24 hr is seen in Table I. The small loss of threonine and serine was in the range expected for hydrolytic destruction and, in any case, less than the amount of sulfonic acid present which, when calculated, represented less than 0.15 residue of cysteic acid/molecule of glucagon. The ninhydrin-positive material cochromatograming with cysteic acid in the sulfite-treated peptide may result from a minor cysteine containing impurity in the preparation but was not further characterized since it was present in trace amounts only.

Lysozyme contains 13.2% unsubstituted hydroxyamino acid in addition to 8 cysteine residues all of which are involved in disulfide bridges. Direct treatment of lysozyme under standard reaction conditions results in the appearance of cysteic acid, although the serine and threonine content remained unchanged. This may be due to elimination–addition prior or subsequent to sulfitolysis of the disulfide bridges (Swan, 1957; Donovan, 1967; Bohak, 1964).

Following reduction and S-carboxymethylation, amino acid analysis indicated that better than 80% of the cysteine residues were alkylated. The data following elimination–addition (Table II) support the conclusion that the appearance of cysteic acid was due to reaction at S–S or SH loci and not from serine or threonine residues. *S*-Carboxymethylcysteine values were essentially unchanged following exposure to sulfite.

Phosvitin, an unusual phosphoprotein from egg yolk, contains more than 55% hydroxyamino acids, predominantly serine. Since nearly every serine residue is phosphorylated (Mecham and Olcott, 1949), the protein has a very high net negative charge and preferably binds substantial amounts of divalent cations due to its high charge density.

The sulfite addition reaction with this phosphoprotein was studied over a range of pH, sulfite concentration, and time. The results of reaction at pH 12.8 and 0.2 M sulfite concentration are summarized in Table III. The correlation between the loss of serine and the appearance of cysteic acid as a function of time under the determined optimal reaction conditions is illustrated in Figure 2. The constant values remaining for serine and cysteic acid after a 24-hr reaction time coupled with the fact that the protein product was essentially free of phosphate indicates that those serines remaining were probably unsubstituted in the native protein.

The protein product,[2] containing nearly 55% cysteic acid exhibits a markedly altered optical rotatory dispersion (ORD) spectrum as compared to that of native phosvitin (Figure 3).

[2] Referred to subsequently as sulfvitin.

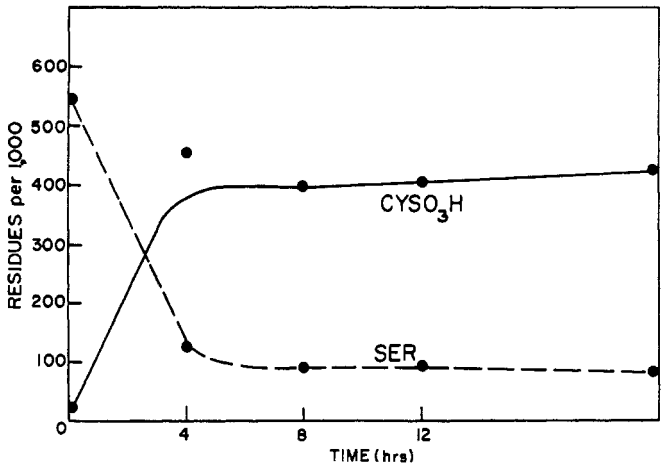

FIGURE 2: Time course of the reaction of phosvitin with alkaline sulfite.

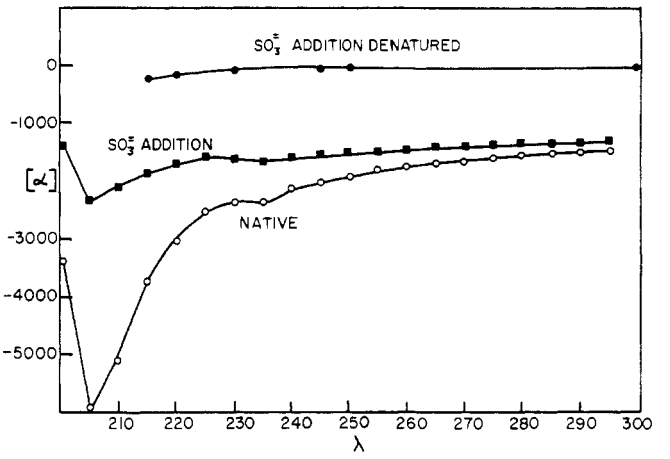

FIGURE 3: ORD spectra of native phosvitin, sulfvitin, and denatured sulfvitin in 5 M guanidinium chloride. The spectra were obtained in 0.01 M Hepes buffer (pH 6.9) except for the denatured sample.

The sulfite reaction product appears to have some residual ordered structure, although it is apparent that the overall negative contribution to the molecular rotation due to L-serine has been lost. A similar study with bovine serum albumin suggested minimal formation of denatured structures (see Figure 4).

The sulfvitin was prepared on a preparative scale (250 mg) for further characterization of the sulfite adduct. Following acid hydrolysis, the sulfite adduct was recovered after chromatography on Dowex 50 with pyridine–acetate. Amino acid analysis revealed that the sample was completely free of all amino acids except for a single peak which eluted at a position corresponding to cysteic acid on a standard chromatogram. A second sample (*ca.* 0.1 μmole) was similarly chromatographed following admixture with standard cysteic acid. The mixed sample appeared on the chromatogram as a single symmetrical peak.

Thin-layer cochromatography with standard cysteic acid was also demonstrated in the following three solvent systems: ethanol–water (70:30, v/v), 1-propanol–water (70:30, v/v), and methyl ethyl ketone–pyridine–water–acetic acid (70:15:15:2, v/v). Cysteic acid had an R_F value of >0.4 in each of these and the characteristic ninhydrin color was observed for the sulfvitin-derived sample. A comparison of the acid stability of serine *O*-phosphate to that of the sulfite adduct showed the former to be essentially completely cleaved to serine and inorganic phosphate under the standard hydrolytic conditions employed. These data eliminate the possibility that the product of sulfite treatment is a phosphate ester of serine and supports the identity of the adduct as cysteic acid.

A sample of cysteic acid isolated from phosvitin following elimination–addition, hydrolysis, and Dowex 50 chromatography was reacted with the trifluoroacetamide. The resulting Me_3Si derivative was examined by gas–liquid chromatography and shown to behave identically with a standard Me_3Si derivative of cysteic acid. In addition, symmetrical peak enhancement of standard Me_3Si–cysteic acid was demonstrated by addition of the sulfvitin derived Me_3Si derivative and chromatography of the mixture. These observations confirm

TABLE II: Effect of Alkali Sulfite on S-Carboxymethylated Lysozyme.[a]

Amino Acid[b]	Lit. Values[c]	Zero Time[d]	24-hr Elimination–Addition[d]
Lysine	46.5	43.4	40.9
Histidine	7.7	8.0	7.9
Arginine	85.2	85.2	85.9
Aspartic	162.7	159.7	159.0
Threonine	54.2	51.5	55.1
Serine	77.5	72.9	78.8
Glutamic	38.5	35.5	43.7
Proline	15.5	16.6	19.8
Glycine	102.4	105.5	114.8
Alanine	102.4	93.1	101.2
Cystine	62.0	15.3	12.7
Valine	46.5	36.6	41.3
Methionine	15.5	14.2	15.1
Isoleucine	46.5	38.7	42.3
Leucine	62.0	59.7	64.7
Tyrosine	23.0	21.8	24.9
Phenylalanine	23.2	21.0	20.7
Cysteic			
S-Carboxymethyl-cysteine		36.2	33.7

[a] Amino acid analysis of *S*-carboxymethyllysozyme before and after 24-hr treatment with alkaline sulfite. [b] Values are in residues per 1000 residues; corrected for tryptophan content. [c] Canfield and Liu (1965). [d] Average of two analyses.

TABLE III: Effect of Alkali Sulfite on Phosvitin.[a]

Amino Acid[b]	0 hr	8 hr	24 hr
Serine	515	71.4	59.1 (−455.9)
Threonine	35	20.0	17.5
Cysteic		407.2	432.8
Phosphate	445	35	<15

[a] Effect of alkaline sulfite on the hydroxyamino acid content of phosvitin. [b] Residues per 1000 residues. The 24-hr product contained less than 0.3% phosphate (limit of detection).

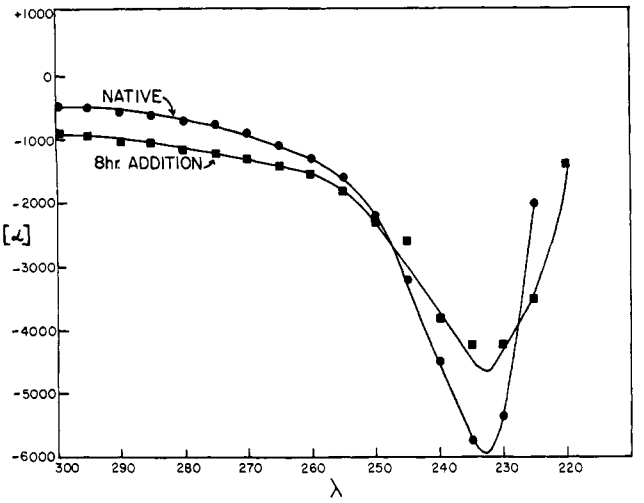

FIGURE 4: ORD spectra of bovine serum albumin before and after 8 hr treatment with alkaline sulfite. Solvent is 0.01 M Hepes buffer (pH 6.9).

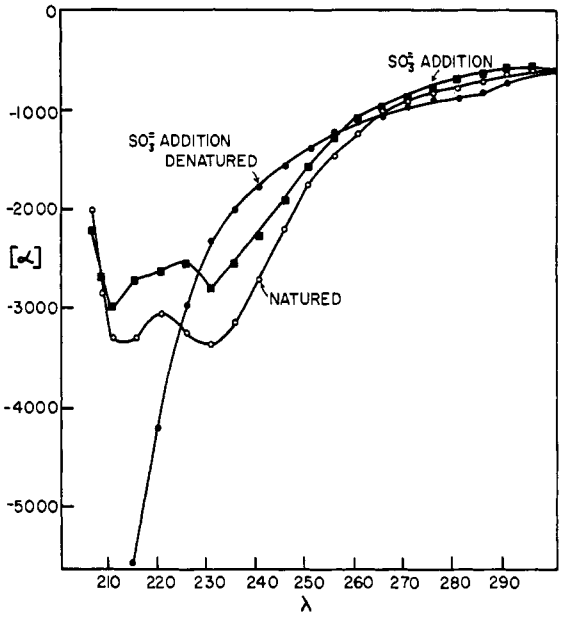

FIGURE 5: ORD spectra of α-casein before and after elimination–addition. Solvent is 0.01 M Hepes buffer (pH 6.9). The sulfite addition product was examined in 5 M guanidinium chloride (denatured spectrum).

the conclusion that the elimination–addition product from sulfvitin is cysteic acid.

α-Casein was reacted with alkaline sulfite under the described experimental conditions. The sample of α-casein employed in these studies contained 9 moles of phosphate, 15 serine, and 6 threonine residues per mole based on a molecular weight of 27,000 (McKenzie, 1967). Results are summarized in Table IV.

The obvious direct correlation between serine loss and cysteic acid formation accompanied by a stoichiometric decrease in phosphate as a function of reaction time, indicates that the phosphate is esterified primarily, if not exclusively to serine, an observation entirely consistent with reported partial sequence data (Österberg, 1964).

The integrity of the polypeptide backbone following the sulfite reaction and the effect of the conversion of serine phosphate to cysteic acid on the primary structure were assessed by examining the optical rotatory behavior of the modified protein. The spectra, shown in Figure 5, suggest retention of nearly native conformation. If extensive peptide bond cleavage had occurred during the reaction, a spectrum more characteristic of the denatured state might have been seen. Molecular weight determinations were not performed. The ORD spectrum of the product following denaturation exhibits typical random coil behavior.

The shift toward a more positive rotation apparent in the spectra of the sulfite-treated sample is probably due to a loss of optical purity at individual reaction sites.

DFP-trypsin was reacted with alkaline [^{35}S]sulfite for 18 hr at room temperature. The [^{35}S]trypsin was purified by chromatography in 0.4 M NaCl–8 M urea on G-15 Sephadex (2.5 × 60 cm) and the labeled trypsin was subjected to acid hydrolysis. The resulting cysteic acid, approximately 0.7 mole/mole of trypsin, was purified by Dowex 50 chromatography and contained all of the incorporated ^{35}S label. Specific identification of the labeled cysteic acid as arising from the active site serine was not undertaken.

The results of a kinetic study with porcine costal cartilage proteoglycan are summarized in Figure 6. There is an excellent correspondence between serine loss and the appearance of cysteic acid as a function of time. It is also evident that the reaction is complete in 8 hr and that the sum of serine and cysteic acid is constant and equal to the initial total concentration of serine in the complex. The trough apparent early in

TABLE IV: Effect of Alkali Sulfite on α-Casein.[a]

Amino Acid[b]	0 hr	8 hr	Net
Serine	58.5	30.6	−27.9
Threonine	22.6	19.4	−3.2
Cysteic	0.1	26.4	+26.3
Phosphate	30	<3[c]	−27

[a] Effect of alkaline sulfite on the hydroxyamino acid content of α-casein. Values are substantially unchanged after 24-hr reaction time. [b] Residues per 1000 residues. [c] Limit of detection.

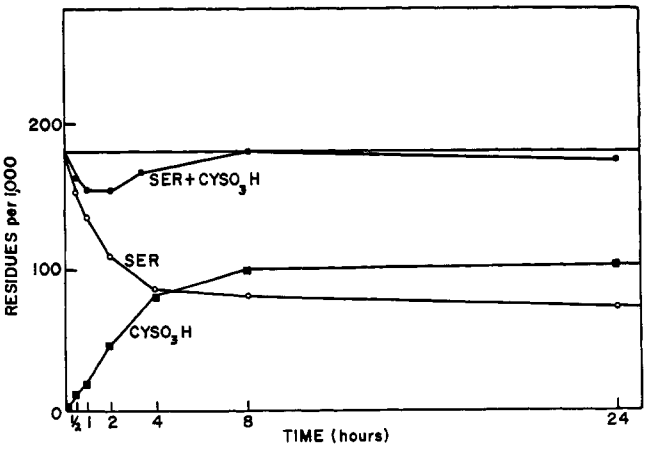

FIGURE 6: Time course of the reaction of cartilage proteoglycan with alkaline sulfite.

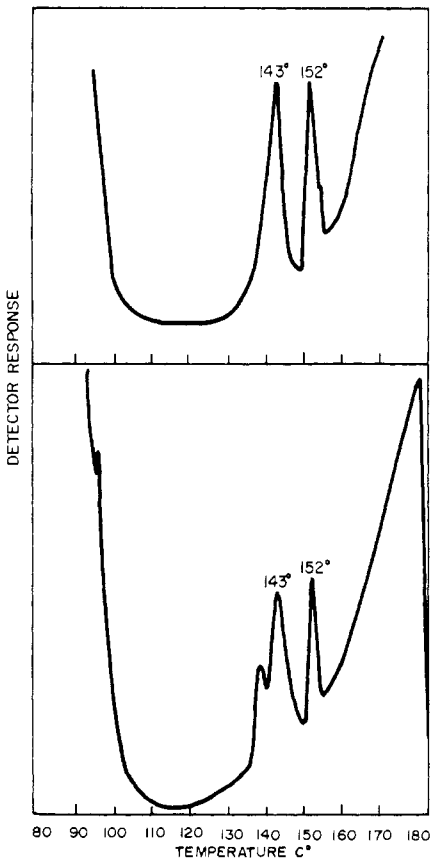

FIGURE 7: Gas chromatographic separation of sulfonic acid products resulting from treatment of bovine submaxillary mucin with alkaline sulfite. Based on peak heights formed from standard samples, saccharide substitution on serine and threonine residues is nearly equivalent and represents about 75% of the available sites.

TABLE V: Effect of Alkali Sulfite on Bovine Submaxillary Mucin.[a]

Amino Acid	0 hr	4 hr	8 hr	Net
Lysine	7.1	6.3	6.6	
Histidine	4.6	4.5	3.5	
Arginine	39.7	38.3	38.8	
Aspartic	23.0	23.2	24.3	
Threonine	143.3	123.2	105.2	−37
Serine	191.5	136	98	−93
Glutamic	60.5	62.4	63.9	
Proline	105.3	105	106.5	
Glycine	183.3	197.6	201.6	
Alanine	122.1	123.7	125.8	
Valine	64.2	61.8	61.6	
Isoleucine	14.3	13.2	11.3	
Leucine	36.5	40.2	41.8	
Tyrosine	3.5	3.3	3.7	
Phenylalanine		4.7	4.6	
Cysteic		56.6	102.9	+102.9

[a] Amino acid analysis of bovine submaxillary mucin before and after treatment with alkaline sulfite. 24-hr values were substantially identical with those obtained at 8 hr. Data do not include amino sugars. [b] Residues per 1000 residues excluding amino sugars.

the time course of the reaction indicates a slight lag between β elimination and subsequent addition of the sulfite nucleophile. The detailed characterization of the reaction products is described in the succeeding paper.

Hydrolysis studies of ^{35}S-labeled peptide in 6 N HCl for 24, 48, and 72 hr show no significant decrease in the cysteic acid peak as a function of time, eliminating serine *O*-sulfate as a possible contaminant.

Bovine submaxillary mucin contains 30% hydroxyamino acids, serine and threonine, both of which are extensively substituted with carbohydrate (Nisizawa and Pigman, 1960; Tettamanti and Pigman, 1968). The amino acid composition is enriched in aliphatic residues and there is little or no cysteine. This mucin was chosen for elimination–addition studies of substituted threonyl residues as few models are available which contain carbohydrate substituted exclusively to threonine. The freezing point depressing glycoprotein described by Feeney (DeVries *et al.*, 1970) is an ideal model but was not readily available. The behavior of bovine mucin in the sulfite addition reaction is summarized in Table V. The sum of serine and threonine loss is comparable to the amount of cysteic acid appearing following reaction with sulfite. These data suggest strongly that both glycosylated serine and threonine residues are participating in the elimination–addition reaction since the resulting sulfonic acid products, cysteic acid and 2-amino-3-sulfonylbutyric acid, are not resolved by the amino acid analyzer procedure employed. Approximately 60% of the amino sugar was lost after 8 hr, a value consistent with the reported degree of saccharide substitution present.

A preparative sample of alkaline sulfite-treated bovine mucin was hydrolyzed, the sulfonic acid fraction isolated by Dowex 50 chromatography and the Me_3Si derivatives prepared.

A typical chromatographic resolution of derivatives is seen in Figure 7 which exhibits two major peaks with maxima at 143 and 152°. A small peak appearing at a lower temperature was not identified. The peak at 143° was enhanced in a symmetrical fashion by addition of standard Me_3Si–cysteic acid and the peak at 152°, the presumed Me_3Si ester of 2-amino-3-sulfonylbutyric acid, was likewise enhanced by addition of standard Me_3Si–homocysteic acid.

Discussion

The general utility of the elimination–addition reaction for structural studies of glycoproteins, phosphoproteins, and proteoglycans lies in its nearly quantitative nature, lack of side reactions and ability to use ^{35}S-labeled sulfite, permitting pertinent reaction sites to be readily labeled. In conjunction with the gas–liquid chromatographic separation of the sulfonic acid reaction products and the inherent base stability of the other types of carbohydrate–peptide linkages, one can readily quantitate the nature of the covalent linkages and determine the relative degree of substitution of carbohydrate to serine and to threonine within a single polypeptide chain. Since both cysteine and cystine can give rise to dehydroalanyl residues under the reaction conditions employed (Cecil, 1963), S-carboxymethylation should be performed if appropriate.

Experiments with cartilage proteoglycan indicated that a distinct lag occurred in the early time course of the reaction which can most readily be interpreted as a two-step mechanism where the formation of cysteic acid lags somewhat behind the β elimination.

Preliminary experiments suggested that the β elimination

of esterified phosphate was incomplete in the absence of sulfite and led to consideration of a displacement mechanism as an alternate to the assumed elimination–addition. Such a reaction path would result in the initial stereospecific formation of L-cysteic acid from L-serine, while β elimination and subsequent α–β addition of the sulfite nucleophile to the unsaturated derivative would destroy the native asymmetry at the α-carbon atom resulting in the formations of DL-cysteic acid within the polypeptide. However, at the pH required for β elimination, racemization may occur with loss of optical purity at the α-carbon atom. The lag in appearance of cysteic acid combined with the lack of significant effect of sulfite concentration on the reaction rate argues against a direct displacement mechanism. The rate-limiting step appears to be the sulfite addition as illustrated in Figure 8. The base abstracts the proton from the α-carbon atom with subsequent release of a nucleophile from the β-carbon atom, be it phosphate, monosaccharide, oligosaccharide, or polysaccharide chain, resulting in the formation of a double bond. The methylene group is attacked by the nucleophilic sulfur resulting in adduct formation.

It has been demonstrated that the nature of the eliminated group from substituted 3-hydroxyamino acids was not a primary rate-determining factor and the additional methyl group on threonine has relatively little effect on the rate of β elimination (Vercellotti *et al.*, 1970). In general, for β elimination of seryl and threonyl residues to take place at a significant rate it is essential that the amino and carboxyl group be substituted (Derevitskaya *et al.*, 1967).

The ORD spectra of sulfite-treated phosvitin and α-casein show a shift toward a more positive rotation, which is consistent with loss of optical purity on β elimination followed by the production of an internal racemate with respect to cysteic acid. This effect cancels the contribution of L-serine to the molecular rotation, and is directly proportional to the cysteic acid composition of the resulting protein. It should be noted that the optical properties of the cysteic acid formed in these reactions were not directly assessed.

In the cartilage proteoglycan and in bovine mucin, β elimination was apparently complete at a lower pH than was required for quantitative conversion to sulfonic acid derivatives. This result emphasizes the necessity for determination of optimal reaction conditions prior to preparative studies. This is also necessary to minimize the cleavage of peptide bonds at the elevated pH ranges employed. In addition, suitable material balance data should be obtained to ensure that the isolated, cysteic acid containing peptides are representative of the starting material.

The alkali-catalyzed hydrolysis of peptide bonds has been studied in some detail and the most susceptible bonds identified as Gly-Gly and Gly-Ser (Jarboe *et al.*, 1971). The rate of hydrolysis of model peptides at pH 12.6 indicates that fewer than 1 bond/100 will be hydrolyzed under the usual conditions employed for the elimination–addition reaction (pH 11.6, 0.2 M sulfite). It is apparent, however, that the elimination reaction will be accelerated at higher pH since the proton on the α-carbon atom will be more readily removed. Retention of near native structure by casein and phosvitin argues against extensive cleavage although hydrolysis at dehydroalanyl loci prior to addition of sulfite may occur. Should this be extensive, the generated cysteic acid residues would be terminal, a result not observed in preliminary studies on several modified proteins. It cannot be stated unequivocally that peptide-bond cleavage does not occur but the magnitude does not seem significant for the compounds studied (Stern *et al.*, 1971).

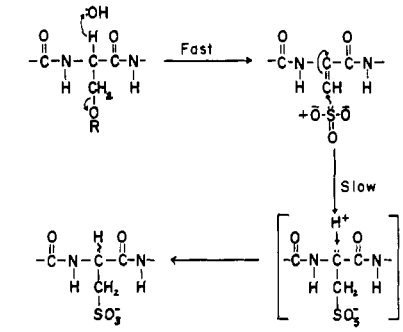

FIGURE 8: Proposed mechanism for elimination–sulfite addition reaction.

Since the other types of covalent bond between carbohydrate and protein, *O*-glycosidic linkage to hydroxylysine and hydroxyproline and asparaginyl *N*-glycosides, are stable under the conditions employed this reaction provides a method of differentiating the nature of the carbohydrate–peptide bonds in complex proteins. In combination with the gas–liquid chromatographic separation of the Me_3Si derivatives of cysteic and 2-amino-3-sulfonylbutyric acids, the ratio of serine to threonine *O*-glycosidic bonds can be readily determined for a given glycoprotein. The separation and quantitation is necessary only when both seryl and threonyl bond are found or known to exist in a protein and the proportion of each involved is at issue. In any case, the use of ^{35}S-labeled sulfite will allow labeling of pertinent sites which can be further defined as sequence information is developed.

The effects of reaction conditions on the carbohydrate moiety released has not been thoroughly studied and some alkali-catalyzed degradation may occur. However, chondroitin 4-sulfate chains released from cartilage proteoglycan are monodisperse (Woodward *et al.*, 1972) and still retain the terminal xylose moiety.

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Isolation of the Peptide Core of Costal Cartilage Chondroitin 4-Sulfate Proteoglycan†

D. L. Simpson and E. A. Davidson*

ABSTRACT: The polypeptide core of the proteoglycan extracted from porcine costal cartilage has been isolated after β elimination and [^{35}S]sulfite addition. The attachment sites of chondroitin 4-sulfate chains to serine in the polypeptide backbone were specifically and quantitatively labeled by conversion to [^{35}S]cysteic acid within the structure. [^{35}S]Cysteic acid, isolated from a preparative hydrolysate, was identified by several independent chemical and chromatographic procedures. The major cysteic acid containing peptide, representing about 75% of the total protein, was resolved from a collagen-like protein on Dowex 1 or DEAE-Sephadex-A-50. The polypeptide was homogenous on Sephadex chromatography and acrylamide gel electrophoresis. There was nearly quantitative recovery of galactosamine as chondroitin 4-sulfate chains, free of protein. Collagenase digestion of the proteoglycan eliminated a protein component but the residual amino acid composition and molecular weight distribution of the product suggested that some proteolysis of the peptide core had occurred. Based on the reactions described in the preceding paper, the molecular weight of the saccharide chains recovered correlates well with that calculated from the ratio of galactosamine to cysteic acid after the elimination–addition reaction.

The structure of the major matrix proteoglycan of costal cartilage is that of a polypeptide to which are covalently attached a number of chondroitin 4-sulfate chains (Marler and Davidson, 1965). The linkage between the saccharide chains and the protein core involves the hydroxyl group of serine and an unusual trisaccharide terminating in D-xylose. The structure of the linkage region oligosaccharide has been reported (Lindahl and Rodén, 1966; Rodén and Smith, 1966) but the distribution of chains on the polypeptide core and the properties of the latter have not been completely elucidated.

The lability of the seryl glycosides to alkali elimination has been utilized as the basis for an addition reaction permitting the localization of the substituted serine residues (Simpson *et al.*, 1972). In addition, the introduction of new charged residues (cysteic acid) into the polypeptide permits ready separation from other proteins as well as from the eliminated saccharide chains. This paper describes the isolation, purification, and properties of the polypeptide core of the predominant porcine costal cartilage proteoglycan. A preliminary report has been presented (Hranisavljevic *et al.*, 1971).

Materials and Methods

Chondroitin 4-sulfate proteoglycan was prepared by a modification of the procedure of Sajdera and Hascall (1969) using either 2 M $CaCl_2$ or 4 M guanidinium chloride as extractant.

† From the Department of Biological Chemistry, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received December 28, 1971.* This work was supported by Public Health Service Grant AM12074.

* To whom all correspondence should be addressed.