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PURIFICATION AND PROPERTIES OF *ARTHROBACTER* NEURAMINIDASE

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Summary

Neuraminidase (EC 3.2.1.18) from an *Arthrobacter* species was purified to homogeneity by conventional procedures (yield approx. 1 mg/l) and was judged to be homogeneous by sodium dodecyl sulfate gel electrophoresis. Gel electrofocusing of neuraminidase revealed 1 major band (85—90%), p*I* 5.35 ± 0.05, and 6 minor bands, whose p*I* ranged from 5.25 to 5.70, and each of which had catalytic activity. *Arthrobacter* neuraminidase is a monomeric glycoprotein of molecular weight 88 000, has an apparent K_m of $7.8 \cdot 10^{-4}$ M for *N*-acetylneuraminlactose, is insensitive to inhibition by *N*-acetylneuraminic acid, and is about 2% carbohydrate by weight. The amino acid composition as well as the galactosamine and glucosamine content was determined. The enzyme can hydrolyze (α, 2—3), (α, 2—6), or (α, 2—8) linkages. The active site of the enzyme appears to be inaccessible, since no inhibition was observed by reagents known to modify sulfhydryl, lysyl, carboxyl, histidinyl, and argininyl residues. In contrast, *N*-bromosuccinimide at a 60-fold molar ratio to enzyme, gave complete inhibition. These results suggest that a tryptophan residue is essential for catalysis.

Introduction

Bacterial neuraminidases (EC 3.2.1.18) from various origins have long been utilized by cell biologists and biochemists in order to selectively hydrolyze sialic acid residues from carbohydrates, gangliosides and glycoproteins (reviewed in refs. 1—3). These investigations have taken advantage of the absolute specificity of this enzyme for sialic acids linked via α-ketosidic bonds.

A manuscript describing the morphological, physiological and biochemical properties of this organism has appeared (Can. J. Microbiol. 23, 1568—1572 (1978)). A subculture of this isolate, designated *Arthrobacter sialophilus*, has been deposited with the American Type Culture Collection (ATCC No. 31253).

Such studies optimally require neuraminidase preparations which do not contain contaminant toxins, glycohydrolases or proteases. There are, however, numerous reports in the literature which indicate that available preparations contain such unwanted enzymatic activities, even after their purification by affinity chromatography [4—7]. Furthermore, most microorganisms which form neuraminidase are pathogens and in point of fact, produce relatively small quantities of the enzyme.

We have recently reported the isolation of a saprophytic Gram positive organism of the genus *Arthrobacter*, which can produce a large amount of an inducible, extracellular neuraminidase. General properties of this enzyme as well as optimal conditions for its induction have been delineated [8]. In order to further our studies on structure-function biochemistry of bacterial neuraminidases [9,10], and to provide a homogeneous source for application to molecular biological investigations, we wish to report its purification to functional homogeneity. A preliminary report of this work has been presented [11].

Materials and Methods

Materials. Reagents used were Bacto-tryptone (Difco Laboratories), yeast extract (Baltimore Biological Laboratories), ammonium sulfate (Schwarz-Mann; enzyme grade), DEAE-cellulose (Bio-Rad, Cellex D), Sephadex G-200 (Pharmacia Fine Chem.), *N*-acetylneuraminlactose (Boehringer-Mannheim), electrophoresis reagents (Bio-Rad Laboratories), colominic acid (Calbiochem), and Ampholine (LKB). All other chemicals were of reagent grade. 'Edible bird's nest' was obtained from a local Chinese grocery store.

Growth of cells. Cultures of *Arthrobacter* [8] were grown on 1% (w/v) tryptone/0.5% (w/v) yeast extract in 20-l jugs containing 12.5 l of medium with aeration at 30°C for 24 h. After centrifugation, the cells were washed first with sterile 0.9% NaCl and then suspended overnight in a minimal salt medium 58 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, 1.28 g $MgSO_4$ in 1 l deionized water. This salt treatment was required to enhance enzyme formation [8]. Induction was carried out for 6 h at 30°C by diluting the salt-shocked cells to a final salt concentration of 2% with acid-treated 'bird's nest' (final concentration 0.40 mg/ml); this inducer was prepared as described previously [8].

Assay. The standard neuraminidase assay mixture contained 80 μmol of citrate/phosphate buffer (pH 6.0), 2.0 mg of *Collocalia* mucoid [12], and enzyme in a final volume of 1.0 ml. Incubations were carried out at 37°C. Aliquots of 0.20 ml were withdrawn at 5 and 10 min, and the *N*-acetylneuraminic acid (AcNeu) was determined essentially as described by Warren [13]. In all experiments, zero time samples were also run. A unit of enzyme activity is defined as that amount which releases 1 μmol/min under the standard conditions. In some experiments, 10 mM sodium acetate buffer (pH 6.0) replaced the citrate/phosphate buffer. Specific activity is expressed as units/mg protein. Protein was determined by the method of Lowry et al. [14], with crystalline bovine serum albumin as the standard.

Gel electrofocusing. The procedure used was a modification of the method described by Wrigley [15]. Neuraminidase was included directly into the gel.

Focusing of the pH gradient was performed at 4°C at a constant current of 1 mA/gel until a potential of 400 V was reached, which was then maintained for a further 1 h. The gel was fixed in 12.5% trichloroacetic acid, washed with water, stained with Coomassie Blue (0.1% in 45% ethanol and 10% glacial acetic acid), and destained in 7% acetic acid. Enzyme activity and pH measurements were determined by slicing a duplicate gel into 1-mm sections using a Bio-Rad slicer, and suspension of each section in 1.0 ml water overnight at 4°C. The pH was determined using a Radiometer pH meter equipped with an expanded scale.

Gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at pH 8.5 in 6% gels [16] and stained with Coomassie Blue, as described previously [8]. Gels were stained for neuraminidase activity using methoxyphenol-*N*-acetyl-α-neuraminate [17]. SDS polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [18]. Protein standards were cross-linked marker proteins obtained from Gallard-Schlesinger Chemical Mfg. Corp. The monomer molecular weight was 14 300.

Amino acid analysis. Samples for amino acid analysis were dialyzed extensively against distilled, deionized water, and hydrolyzed in evacuated glass tubes with 6 M HCl for 22 h at 110°C. Analyses were performed in duplicate on a Technicon NC-2P amino acid analyzer.

Purification procedure for neuraminidase

A summary of the steps in purification of neuraminidase is given in Table I.

Preparation of induction filtrate (Step I). The induction filtrate from *Arthrobacter* cultures was obtained by continuous centrifugation using a Sorvall SS 34 rotor (Step I). All additional procedures were performed at 4°C.

$(NH_4)_2SO_4$ fractionation (Step II). To the cell-free induction filtrate, solid $(NH_4)_2SO_4$ was added slowly to 0.80 saturation (561 g/l). The mixture was stirred for an additional 30 min, and then centrifuged at 10 000 × *g* for 15 min. The pellet was dissolved in a minimal volume of 0.01 M citrate phosphate buffer (pH 6.0) containing 10^{-4} M phenylmethylsulfonylfluoride (Buffer A). The solution was then dialyzed against a 20-fold excess of Buffer A with several changes. The undissolved material was removed by centrifugation, and the supernatant was decanted and saved (Step II).

TABLE I

PURIFICATION OF *ARTHROBACTER* NEURAMINIDASE

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
I. Induction filtrate	23 270	7035	11 073	0.626	100
II. $(NH_4)_2SO_4$ precipitation (0—80%) *	500	6808	1 740	3.91	96.8
III. DEAE-cellulose chromatography	1 120	6184	549	11.27	87.9
IV. Ultra-filtration (PM-10)	4.2	5682	203	27.94	80.8
V. Sephadex G-200 chromatography	172	3547	73.3	48.4	50.4
VI. 2nd $(NH_4)_2SO_4$ precipitation					
Precipitate I	5.05	1485	11.6	110	18.3
Precipitate II	4.1	857	9.5	90.0	12.2

* Purification steps II-VI were carried out at 4°C.

DEAE-cellulose chromatography (Step III). The sample from the previous step was passed over a column of DEAE-cellulose (2 × 80 cm) that had been equilibrated with Buffer A. Under these conditions, neuraminidase was not absorbed, whereas 33% of the total protein remained tightly bound.

PM-10 diaflo ultrafiltration (Step IV). The active fractions obtained from the previous step were pooled and concentrated by pressure filtration (nitrogen) using an Amicon ultrafiltration apparatus with a Diaflo PM-10 membrane. This step gave a 2.5 fold purification as a result of the loss of approx. 60% of the Lowry-positive material, which is presumably low molecular weight peptides present in the acid-treated 'bird's nest'.

Sephadex G-200 chromatography (Step V). A column (2.5 × 100 cm) was prepared and washed with Buffer A. The enzyme solution was layered onto the column, and the enzyme was eluted as a sharp activity peak (Fig. 1). The presence of a single symmetrical activity peak is in marked contrast to the multiple activity peaks often observed with other bacterial neuraminidases [10,19—21]. Fractions containing enzyme activity were combined and concentrated by pressure filtration as described above.

$(NH_4)_2SO_4$ precipitation (Step VI). To remove the remaining impurities, saturated $(NH_4)_2SO_4$ was added dropwise to Fraction V until the material was slightly cloudy, and the mixture was allowed to stand overnight at 4°C. The precipitate was collected by centrifugation in a Sorvall RC 2B at 27 000 × *g* for 1 h. It was dissolved in Buffer A (precipitate I). Additional saturated $(NH_4)_2SO_4$ was added to mother liquor as described above, and the precipitate collected by centrifugation (precipitate II). Both preparations were extensively dialyzed against Buffer A and stored at —20°C. Under these conditions, the purified enzyme has remained stable for over a year. The yield of enzyme was

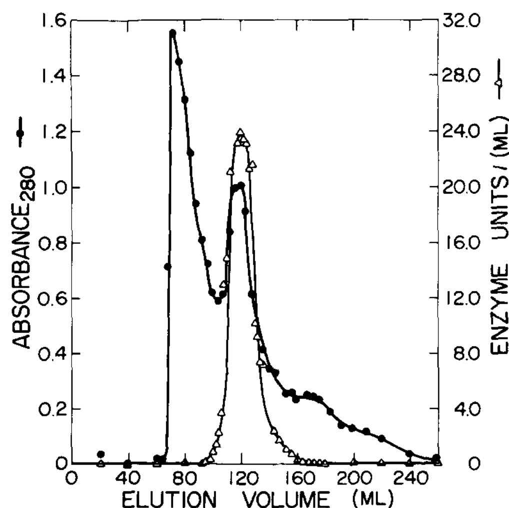

Fig. 1. Purification of neuraminidase by gel filtration on Sephadex G-200. A Sephadex G-200 column (2.5 × 100 cm) was equilibrated with Buffer A, and used at 4°C. A solution of the enzyme in Buffer A (4.2 ml) containing 203 mg of protein was applied to the column. The column was eluted with Buffer A at a flow rate of 12 ml/h, and the fraction volume was 2 ml. The triangles indicate the activity of neuraminidase as a function of the elution volume; the circles represent the protein as a function of elution volume.

approximately 1 mg/l of induction filtrate. The final preparation (purified 174-fold) had a specific activity of 110 units/mg of protein. When the *Arthrobacter* enzyme (precipitate I) was assayed under conditions of low ionic strength (0.01 M sodium acetate, pH 6.0) it exhibited a specific activity of 225.

Results

Analysis of purity. Although precipitates I and II obtained by ammonium sulfate crystallization differed slightly in their final specific activities each preparation gave identical analytical gel patterns. The purified protein, after polyacrylamide gel electrophoresis under standard non-denaturating conditions displayed one major protein band and a closely affiliated minor band. For localization of enzyme activity, unstained replicate disc gels were cut into 1-mm sections and incubated overnight in Buffer A at 4°C. Neuraminidase was associated with each of the major and minor bands and, in addition, a third trace activity band was also observed. In a separate analysis using the specific neuraminidase activity stain, *o*-methoxyphenol-*N*-acetyl-α-neuraminate [17], the enzyme was also localized as coincident with the major and two minor bands. Densitometer tracings of the stained gel at 550 nm indicated over 95% of the applied protein in association with the major band. An SDS polyacrylamide gel electrophoresis analysis of the homogeneous enzyme is shown in Fig. 2. In contrast to the results described above, with 50 μg of protein/gel, only a single protein band was observed. Similar results were obtained with 10% gels. These experiments are consistent with the conclusion that the neuraminidase has been purified to greater than 95%.

Gel electrofocusing. The homogeneous enzyme was also analyzed by gel electrofocusing. The results of a pH profile between pH 4 and pH 6 are shown in Fig. 3. Control experiments, using a pH gradient between pH 3 and pH 10, indicated that all of the protein present in the preparation migrated between the pH 4—6 region. As shown in Fig. 3, with a sample size of 10 μg/gel, one major Coomassie Blue band and 6 minor bands were observed. To determine if these protein bands reflected neuraminidase activity, an unstained duplicate gel was sliced into 1 mm sections, suspended in 1.0 water, allowed to stand at 4°C overnight, and was assayed for catalytic activity. Each band manifested neuraminidase activity. Densitometer tracings of the stained gel indicated that the major band contained between 85—90% of the protein applied, with the remaining material distributed among the minor bands. The isoelectric point of the major enzyme form was 5.35 ± 0.05, and the isoelectric points for the minor isofunctional forms ranged from 5.25 to 5.72.

Electrofocusing analyses similar to these were also directly performed on the induction filtrate. Such gel protein-activity patterns were similar to those found for the homogeneous enzyme. This suggests that the presence of multiple activity forms in the homogeneous enzyme is not a result of the purification procedures.

Molecular weight determination and subunit composition. In order to investigate the subunit structure of neuraminidase, the molecular weight of the homogeneous enzyme was determined by SDS polyacrylamide gel electrophoresis [18]. A molecular weight of 89 000 was estimated for the *Arthrobacter*

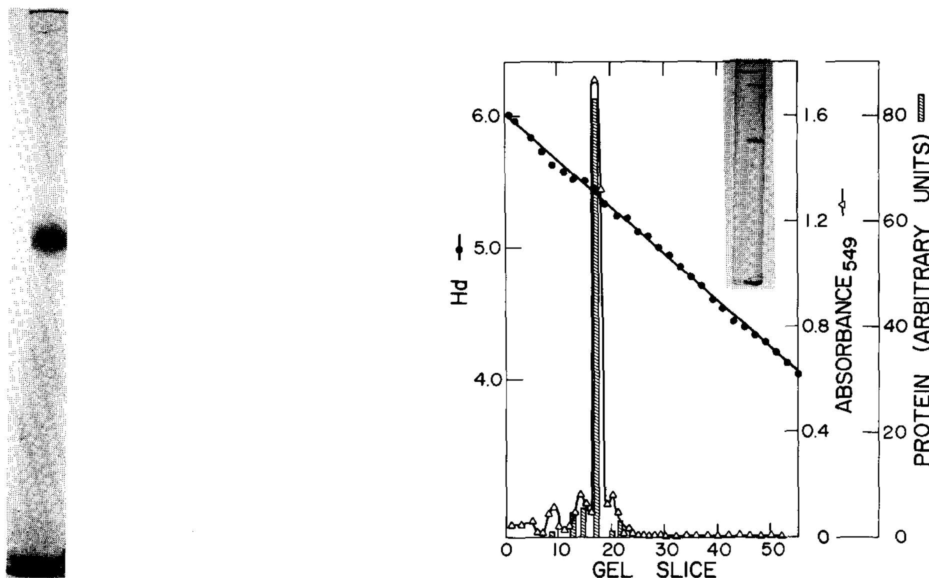

Fig. 2. Gel electrophoresis of neuraminidase in the presence of 1% SDS/1% β-mercaptoethanol. Neuraminidase, precipitate I, (50 μg) was applied to a 5% polyacrylamide gel, subjected to electrophoresis, and stained as described under Materials and Methods.

Fig. 3. Gel electrofocusing of neuraminidase. Neuraminidase (precipitate I, 10 μg) was subjected to gel electrofocusing at 4°C in 8.3% gels containing 2% ampholine (pH 4—6). The pH and neuraminidase activity determined as described under Materials and Methods. The triangles indicate the activity of neuraminidase as related to the gel slice; the circles represent the pH as related to the gel slice. The protein pattern was determined by scanning a duplicate Coomassie-Blue-stained gel with a Gilford 2410-S linear transport on a Gilford 240 spectrophotometer, and the area under each peak was determined with the use of a planimeter. Insert — Duplicate gel stained with Coomassie Blue as described under Materials and Methods.

enzyme. We have previously reported that the molecular weight of this neuraminidase obtained by use of a calibrated Sephadex G-150 column was 87 000 [8]. These results affirm the conclusion [8] that this enzyme is a monomeric protein of molecular weight approx. 88 000.

Amino acid analysis. The amino acid composition of the enzyme is shown in Table II. The *Arthrobacter* enzyme contains significantly more acidic amino acids than basic ones, which is consistent with its acid isoelectric point determined by gel electrofocusing. It also contains two disulfide bonds and one free cysteinyl residue. The presence of relatively large amounts of proline residues suggests that the *Arthrobacter* neuraminidase may have little helical structure. The *Arthrobacter* enzyme also contains a small number of glucosamine and galactosamine residues and is about 2% by weight neutral sugars. Therefore, neuraminidase is a glycoprotein.

Linkage specificity. Neuraminidases isolated from other bacterial organisms hydrolyze the (α-2,3), (α-2,6) and (α-2,8) glycosidic linkage [1,2]. In order to determine the linkage specificity of the *Arthrobacter* enzyme, the enzyme was incubated with colominic acid or *N*-acetylneuraminlactose. The former is a homopolymer of sialic acid linked via α-2,8-linkages, and the latter is a mixture

TABLE II

AMINO ACID ANALYSIS OF *ARTHROBACTER* NEURAMINIDASE

22-h hydrolysis at 110°C as described under Materials and Methods. The values given are an average of 2 separate hydrolyses, with 2 determinations for each.

Amino acid	Residues per 88 000 daltons
Threonine	83
Serine	62
Glycine	76
Alanine	120
Valine	69
Methionine	10
Isoleucine	21
Leucine	43
1/2-Cystine	5 *
Cystein	1 **
Aspartic acid	100
Glutamic acid	52
Lysine	9
Histidine	22
Arginine	35
Tyrosine	27
Phenylalanine	31
Tryptophan	10 ***
Proline	52
Glucosamine	2 †
Galactosamine	5 †
Total residues	834
Neutral sugar	2% ††

* Oxidation with dimethyl sulfoxide [24].
** Determined using 5,5′-dithiobis (2-nitrobenzoic acid) [25].
*** Determined spectrophotometrically [26].
† Determined by amino acid analysis [27].
†† Determined by the anthrone method [28].

of the α-2,3- and α-2,6-isomers. The total amount of sialic acid in each of these substrates was determined after mild acid hydrolysis using 0.025 M H_2SO_4 at 80°C for 1 h. The results of incubating the enzyme with these substrates are

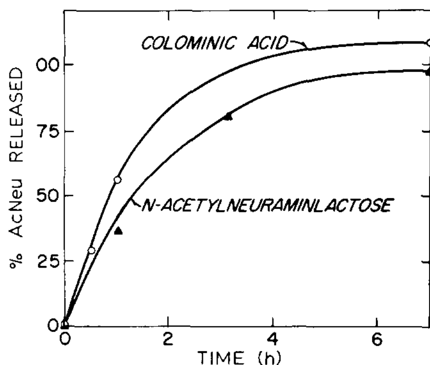

Fig. 4. Determination of the linkage specificity of neuraminidase. The enzyme was incubated with either colominic acid ($2.3 \cdot 10^{-5}$ M) or *N*-acetylneuraminlactose ($2.5 \cdot 10^{-4}$ M) at 37°C, aliquots were removed at indicated times, and AcNeu determined as described by Warren [13]. The total AcNeu in each substrate was determined after mild acid hydrolysis using 0.025 M H_2SO_4 at 80°C for 1 h.

TABLE III

TREATMENT OF HOMOGENEOUS NEURAMINIDASE WITH GROUP-SPECIFIC REAGENTS

Enzyme incubated with indicated inhibitor at room temperature for 20 min except for *O*-methylisourea (3 days), an aliquot removed, and assayed for neuraminidase activitiy as described under Materials and Methods.

Reagent	[Inhibitor] / [enzyme]	pH	% inactivation
N-bromosuccinimide	10 000	8.3	100
	100	5.0	100
	60	5.0	100
Iodoacetamide	10 000	8.3	0
5,5-Dithiobis (2-Nitrobenzoic acid)	2 000	8.3	0
2,4,6-Trinitrobenzene-sulfonic acid	10 000	8.3	11
O-methylisourea	1 900	8.5	0
Dansyl chloride	100	8.3	22
1-Ethyl-(3-diaminomethylpropyl-) carbodiimide *	1 500	4.5	0
2,3-Butanedione	1 900	8.5	0
Diethyl pyrocarbonate	1 700	6.0	0

* Glycene methyl ester and glycinamide used as nucleophiles.

shown in Fig. 4, and indicate that the *Arthrobacter* enzyme can release all of the sialic acid found in these compounds. Therefore, this enzyme is non-specific for positional isomers of α-*N*-acetylneuraminylketosides.

Kinetic properties. As reported earlier [8] the partially purified enzyme showed typical Michaelis-Menten kinetics; the apparent K_m for *Collocalia* mucoid was 2.08 mg/ml and for *N*-acetylneuraminlactose was $3.3 \cdot 10^{-3}$ M. These constants did not change when determined with the homogeneous enzyme. However, when the enzyme was assayed under conditions of low ionic strength the K_m for *N*-acetylneuraminlactose decreased to a value of $7.8 \cdot 10^{-4}$ M. Free AcNeu was found to have no effect on enzyme activity when added to the standard assay at a concentration of $3.3 \cdot 10^{-5}$ M. These results are similar to the neuraminidase isolated from *Clostridium perfringens* [21], but contrast to observations for the enzyme from *Vibrio cholerae* [29].

Preliminary active site studies. In order to initiate a study of the amino acid residues at the active site of neuraminidase, a screening process was undertaken covering a wide range of modifying agents. The results of such a study are presented in Table III. Even under conditions of very high inhibitor to enzyme molar ratios, reagents commonly known to modify sulfhydryl, argininyl, histidinyl, lysyl and carboxyl residues had no effect on enzyme activity. Furthermore, dansyl chloride which has been shown primarily to modify amino groups, was only slightly inhibitory. Only *N*-bromosuccinimide gave significant inhibition. Additional studies indicated that this inhibition was pseudo-first order with respect to enzyme, and at a molar ratio of inhibitor to enzyme of 60, enzyme activity was inhibited completely.

Discussion

Neuraminidase was first discovered in influenza virus in 1945 [1] and soon after in *V. cholerae* [1]; since then numerous biological and biochemical

studies have been carried out with partially purified enzyme preparations. Purification of neuraminidases to homogeneity from pathogenic bacterial sources has been handicapped by low yields under growing conditions and by the complexity of cultivation media. By utilization of a saprophytic *Arthrobacter* isolate which secretes an inducible, extracellular enzyme, a relatively simple procedure for producing and purifying its neuraminidase to homogeneity has now been developed. The purity of this enzyme preparation was established by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions, and by gel electrofocusing. Following earlier studies which indicated the presence of approximately 3—5 mg of enzyme per liter of induction filtrate, and the total absence of associated protease and other glycohydrolase activities [8], the final yield approached 1 mg of enzyme per liter. This is in marked contrast to the recent report by Nees et al. [21] on the purification of neuraminidase from *Cl. perfringens*, in which the final yield of enzyme was 0.01 mg of enzyme per liter of growth filtrate. The ease of obtaining large amounts of *Arthrobacter* neuraminidase by the foregoing procedures makes this particular enzyme preparation ideal for biochemical modifications and for use in cell biology studies.

The availability of this homogeneous *Arthrobacter* neuraminidase has enabled us to determine a number of its physical and chemical properties. Our estimate of 89 000 for its molecular weight, based on SDS polyacrylamide gel electrophoresis, accords with the value (87 000) obtained earlier [8] following gel filtration of the partially purified preparation. The concordance of these determinations strongly suggests a single polypeptide chain. This value does not differ markedly from the molecular weight of a cluster of other bacterial neuraminidases [1,2]. The presence of carbohydrate in neuraminidase was first reported for the viral enzyme [1,2]. Our chemical analysis demonstrates that the *Arthrobacter* enzyme is also a glycoprotein containing both glucosamine and galactosamine, and is about 2% neutral carbohydrate by weight. However, gel electrofocusing of the homogeneous enzyme revealed one major and 6 minor catalytic forms. These isofunctional enzymes, in all probability, do not differ significantly in molecular weight, since only a single protein band was observed using SDS polyacrylamide gel electrophoresis. Microheterogeneity has also been observed in the partial purification of neuraminidases from a number of other bacterial sources [10,19—21]. The amino acid composition of the enzyme was determined. In contrast to the enzyme from *Diplococcus pneumoniae* purified earlier in this laboratory and which did not appear to contain any 1/2 cystine, the *Arthrobacter* enzyme contains two cystine and one free cysteinyl residue. Indeed, there appears to be little in common with respect to the amino acid composition of these two proteins [10]. In order to further investigate analogies in the primary sequences of microbial neuraminidases, we have prepared monospecific rabbit antibodies against the homogeneous *Arthrobacter* enzyme. Parenthetically, the homologous antigen-antibody reaction in gel diffusion studies further indicates the homogeneity of the *Arthrobacter* enzyme (Huchzermeier, R., Tanenbaum, S.W. and Flashner, M., unpublished data).

Although the reactions catalyzed by neuraminidase and the substrate specificity of the enzyme have been thoroughly investigated [1,2], little is

known concerning the identity of those amino acids involved in substrate binding or catalysis. Our initial approach, using group-specific reagents to chemically modify essential amino acid residues, is summarized in Table III. Since no inhibition was observed with reagents known to modify sulfhydryl, lysyl, carboxyl, histidinyl, or argininyl residues, the active site of the enzyme appears inaccessible. In contrast, *N*-bromosuccinimide, at a low molar ratio of inhibitor to enzyme, inhibits enzyme activity completely. These results are in keeping with a preliminary report of Bachmayer [30]. Furthermore, we have observed complete protection of the enzyme against inactivation by *N*-bromosuccinimide when the assay was carried out in the presence of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, a known competitive inhibitor of the enzyme (ref. 31 and Wang, P. and Flashner, M., unpublished data). These results suggest that a tryptophan residue is essential for catalysis.

In catalytic properties the *Arthrobacter* enzyme resembles very closely the *D. pneumoniae* or *Cl. perfringens* enzymes. All three enzymes have very similar K_m values for *N*-acetylneuraminlactose, and are insensitive to inhibition by AcNeu. In addition, neither of these enzymes require Ca^{2+} ions for maximum activity, and all are sensitive [22,23] to changes in ionic strength. This is in contrast to the enzyme from *V. cholera*, which responds to inhibition by AcNeu, requires Ca^{2+} ions for activity and is unaffected by ionic strength when assayed in the presence of low molecular weight substrates.

Because of its facile purification after induction in a concentrated minimal replacement medium, taken together with the convergent criteria for homogeneity as presented here, *Arthrobacter* neuraminidase provides a starting point for structure-function studies of this important glycohydrolase. Our preliminary survey of side-chain reactivities implicates tryptophan at the active center, but has yet to reveal the nature of the proton-donating amino acid residue. However, it can be anticipated that continuing probes with this protein and group specific reagents under reaction conditions which perturb its tertiary structure, and the combined use of affinity-labeling or transition state analogs, will provide more information on the nature of the amino acid residues involved in catalysis.

Acknowledgments

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