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Isomorphous Binding of Mercury-Substituted Thiosaccharides to Pertussis Toxin Crystals Yields Crystallographic Phases*

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Abstract

An isomorphous derivative of pertussis toxin crystals was prepared using a 2-α-mercuric analog of *N*-acetyl neuraminic acid in a method analogous to the use of inhibitors labelled with heavy atoms to solve crystal structures of enzymes. This derivative exploits the specific binding between pertussis toxin and terminal sialic acid residues on receptor glycoproteins. Difference Patterson maps yielded heavy-atom sites which refined with good statistics, indicating that the protein probably does not undergo a conformational change on receptor binding. Mercuric analogs of other monosaccharides should be easily obtainable using the same synthetic strategy, suggesting a general method for derivatizing crystals of carbohydrate-binding proteins.

Introduction

Pertussis toxin (PT) is a member of the AB_5 class of bacterial toxins, so named because the 'binding' pentamer translocates a catalytic 'active' subunit to the interior of target cells (Tamura *et al.*, 1982; Tamura, Nogimori, Yajima, Ase & Ui, 1983). It is the largest of the AB_5 toxins with its six subunits totalling 106 kDa in molecular weight and the only known AB_5 toxin with a heterologous *B* pentamer (for reviews see: Li, 1992; Kaslow & Burns, 1992). Consistent with the behavior of other AB_5 toxins, the *B* pentamer enables the transport of the *A* subunit through the membrane of target cells without inserting itself fully into the hydrophobic portion of the lipid bilayer (Montecucco, Tomasi, Schiavo & Rappuoli, 1986).

PT crystals ($P2_12_12_1$, a = 98.4, b = 164.2, c = 195.2 Å; Spangler, Kuljot, Geplak & Keith, 1988; Raghavan, Gotto, Scott & Schutt, 1990; Turner, Fawcett, Sawyer & van Heyningen, 1990) have more than one toxin molecule per asymmetric unit. Isomorphous replacement requires the preparation of a crystal in which very few heavy atoms are bound at identical points in each unit cell without introducing significant conformational changes (Blundell & Johnson, 1976). Heavy-atom sites in the unit cell may then be located with difference Patterson methods (Rossmann, 1960). When crystals contain more than a few bound heavy atoms, difference Patterson maps are not readily interpretable (Dodson & Vijayan, 1971). After surveying over 50 heavy-atom soaking conditions, interpretable difference Patterson maps for PT crystals were not obtained, possibly because of many atoms bound within the asymmetric unit.

Potentially, the metallized analog of any small molecule which binds to a specific site on a macromolecule can be used to prepare an initial derivative with only one or two heavy atoms per molecule. For example, the crystal structure of *Staphylococcus* nuclease was determined with the 5-iodo analog of deoxyuridine, which fulfilled the requirements of isomorphism and specific binding (Arnone *et al.*, 1971; Blundell & Johnson, 1976). If other derivatives are necessary, sites can be located using cross-difference Fourier methods which are more sensitive than difference Patterson methods, but which rely upon initial phases from a first derivative (Stryer, Kendrew & Watson, 1964).

Pertussis toxin interacts specifically with several small molecules, in particular sialic acid, also known as *N*-acetylneuraminic acid (NeuNAc). Affinity chromatography of the serum sialo-proteins fetuin and haptoglobin has been used to purify PT (Sekura, 1985). Free NeuNAc in solution is known to inhibit the agglutination of avian red blood cells by PT, and Witvliet, Burns, Brennan, Poolman & Manclark (1989) have shown that NeuNAc and sialo-proteins specifically inhibit PT binding to fetuin. The best characterized receptor of PT (Brennan, David, Kenimer & Manclark, 1988) is a membrane sialo-glycoprotein of Chinese hamster ovary (CHO) cells whose activity is lost in cell lines in which sialyl transferases are genetically inactivated (Witvliet *et al.*, 1989). Thus sialic acid is an appropriate candidate for the metallo-ligand labelling of PT in crystals.

We have synthesized a compound [(7) in Fig. 1] in which the 2-α-*O*-linkage of the terminal NeuNAc of fetuin is replaced with a thiomercury 2-α linkage which is expected to cause a minimum of steric interference in binding to PT. The mercury-sulfur bond is readily synthesized. The K_d for sulfur-mercury bonds in RS-Hg-SR' compounds is $10^{-40}\,M^{-2}$ (Stricks & Kolthoff, 1953) indicating a greater stability than most metal-organic bonds. This paper will discuss the results

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of our attempts to use 2-α thiomethyl mercuric sialic acid (NeuNAcHg) to derivatize PT crystals.

Materials and methods

Synthesis

All reactions were followed by thin-layer chromatography (TLC) developed with anisaldehyde stain. Except where otherwise noted all chemical reagents were obtained from Aldrich, all solvents from Baker Chemicals, and TLC plates from Analtech. All the reactions in the synthetic scheme (Fig. 1) were run at room temperature. All NMR spectra were taken on Jeol 270 spectrometers. ^{1}H NMR assignments were facilitated by two-dimensional spectra.

Methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid)-onate (5). Commercial preparations of NeuNAc obtained from Sigma (type IV from *E. coli*) were converted to compound (5) as described by Hasegawa, Nakamura & Kiso (1986) and described in the synthetic scheme (Fig. 1). Mercuric compounds are susceptible to degradation under acidic conditions (Negishi, 1980) and were stored in the dark, under argon, at 253 K.

Methyl [5-acetamido-3,5-dideoxy-2-(thiomethyl mercuric)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (6). Compound (6) was prepared by addition of 10 equivalents of sodium methoxide to compound (5) (100 mg, 180 μmol, 1 equivalent) in 20 ml absolute methanol until the solution was brought to pH 10. When reaction was found to be complete by TLC (80:20 $CHCl_3$:MeOH), the solution was neutralized with washed Dowex 50X8 acidic resin. Subsequently 1 equivalent of methylmercury(II) chloride was added to give compound (6) which was purified by preparative silica TLC using the analytical TLC conditions. The compound was then crystallized in the cold (253 K) by evaporation of the concentrated syrup. These crystals are stable for long-term storage. Yield 62%, 63 mg, 112 μmol.

^{13}C NMR (D_2O, 270 MHz, MeOH internal reference): δ = 175.82 ($NHCOCH_3$), 175.12 (C1), 83.93 (C2), 75.43 (C6), 71.14 (C8), 68.56 (C7), 68.12 (C4), 62.091 (C9), 53.89 (C5), 51.74 (CO_2Me), 45.99 (C3), 22.19 ($NHCOCH_3$) and 8.98 ($HgCH_3$).

^{1}H NMR (D_2O, 270 MHz): δ = 3.88 (*s*; 3H; CO_2CH_3), 3.91–3.81 (*m*; 3H; H5 H7 H8), 3.69–3.63 (*m*; 3H; H4 H6 H9), 3.57 (*dd*; 1H; H9′; J = 9.9, 1.8 Hz), 2.87 (*dd*; 1H; H3e; J = 4.62, 13.2 Hz), 2.03 (*s*; 3H; $NCOCH_3$), 2.01 (*dd*; 1H; H3a; J = 12.21 Hz), 0.86 (*t*; 3H; $HgCH_3$; J_{Hg-H} = 89.5 Hz).

5-Acetamido-3,5-dideoxy-2-(thiomethylmercuric)-D-glycero-α-D-galacto-2-nonulopyranosidonic acid (7). NeuNAcHg (7) was synthesized by adding approximately 50 μl of 2 *M* aqueous lithium hydroxide to a solution of 14 mg of compound (6) in 1.5 ml 60:40 MeOH:THF. When the reaction was complete as shown by silica TLC (80:20:0.5 $CHCl_3$:MeOH:TFA) - approximately 2.5 d - the solution was vacuum filtered over Dowex 50X8 acidic resin to a neutral pH. The compound was crystallized by evaporation of a concentrated methanol solution. Quantitative yield, 14 mg, 26 μmol.

^{13}C NMR (D_2O, 270 MHz, MeOH internal reference): δ = 180.46 (C1), 175.22 ($NCOCH_3$), 85.92 (C2), 75.38 (C6), 72.15 (C8), 69.47 (C7), 67.99 (C4), 62.73 (C9), 51.80 (C5), 45.89 (C3), 22.12 ($NCOCH_3$), and 8.92 ($HgCH_3$).

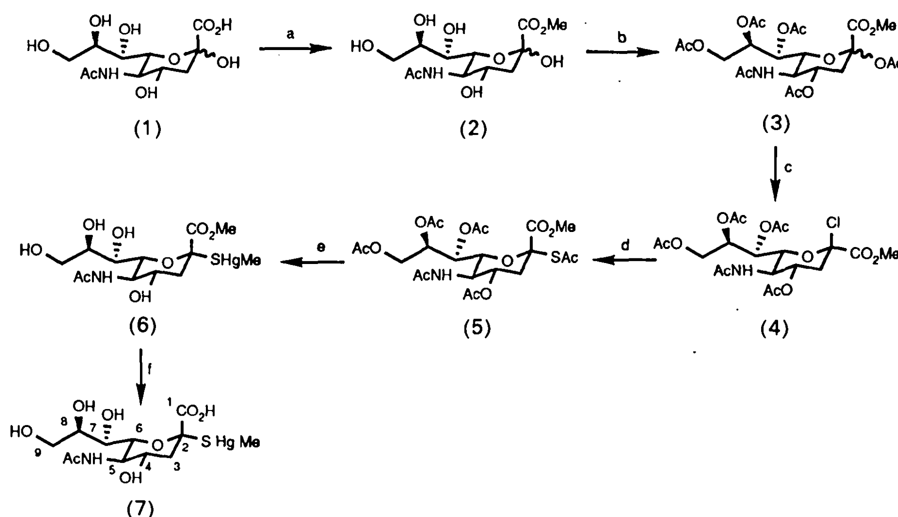

Fig. 1. Synthetic scheme for NeuNAcHg. The acetylation and thioacetate substitution to synthesize (7) [reactions (*a*)-(*d*) were performed as per Hasegawa, Nakamura & Kiso (1986)]. Reaction conditions: (*a*) Dowex 50X8 acidic resin, MeOH, room temperature (RT) (99%); (*b*) $HClO_4$ catalyst, Ac_2O, RT (70%); (*c*) HCl, AcCl, 195 K to RT for 24 h (99%); (*d*) KSAc, 4:1 CH_2Cl_2: DMF, RT (95%); (*e*) NaOMe, MeOH, RT, then CH_3HgCl, MeOH, RT (62%, two steps); (*f*) LiOH, 3:2 MeOH:DMF, RT (100%).

^{1}H NMR (D_2O, 270 MHz): δ = 3.90-3.78 (*m*; 3H; H5 H7 H8), 3.69-3.55 (*m*; 3H; H9 H4 H6), 3.40 (*dd*; 1H; H9; J = 1.8, 10.4 Hz), 2.85 (*dd*; 1H; H3e; J = 4.6, 12.8 Hz), 2.03 (*s*; 3H; CH_3CON), δ = 1.92 (*dd*; 1H; H3a; J = 11.5, 12.9 Hz), 0.83 (*t*; 3H; HgCH_3; J_{Hg-H} = 87.6 Hz).

The chemical shifts of the 3a and 3e H atoms show the products as a pure a stereoisomer at the anomeric center where the S atoms are attached (Dabrowski, Friebolin, Brossmer & Supp, 1979).

Hemagglutination assays

These were performed as per Sekura (1985) with the following specifications: titers were prepared from erythrocyte stock solutions and saccharide stocks in phosphate-buffered saline at various concentrations: lactose (125, 100, 10, 1 m*M*), sucrose (500, 100, 10, 1 m*M*), galactose (500, 100, 10, 1 m*M*), glucose (100, 10, 1 m*M*), *N*-acetylgalactosamine (100, 10, 1 m*M*), *N*-acetylglucosamine (100, 10, 1 m*M*), NeuNAc (50, 10, 1 m*M*), and NeuNAcHg (50, 10, 1 m*M*). Goose or chicken erythrocytes were both found to be useful, although agglutination assays require twice as much PT when chicken blood is used. 50 µl titres of red blood cells in phosphate-buffered saline were incubated for 30 min with one standard activity unit of PT before agglutination activity was assayed.

Protein crystals

PT crystals (Raghavan *et al.*, 1990) were obtained from protein purified from supernatant of cultures of *Bordetella pertussis* (Lederle strain 130) according to the method of Sekura (1985). PT was stored as an ammonium sulfate precipitate at 277 K until crystallized. Protein was resuspended and dialyzed against deionized water, and crystallized *via* vapor diffusion in hanging drops against 250 m*M* sodium chloride, 100 m*M* phosphate buffer (pH 7.4-7.6).

X-ray data collection and analysis

Diffraction data from native crystals were collected on the FAST area detector on beamline X12C at the Brookhaven National Synchrotron Light Source and processed using *MADNES* (Messerschmidt & Pflugrath, 1987) with profile fitting (Kabsch, 1988). Derivatized crystal data sets were obtained by soaking in 10 m*M* NeuNAcHg in stabilizing buffer (100 m*M* phosphate, pH 7.6, 250 m*M* NaCl) for 4 h. The same results were obtained from crystals soaked for times ranging from 2 h to one week. Derivative data were collected on a Siemens area detector with radiation from a rotating copper anode operating at 50 kV, 60 mA. These data were processed with *XENGEN* software (Howard, Nielsen & Xuong, 1985). All data were scaled, merged and phased using the *CCP4* suite of programs provided by Dr Phil Evans (SERC Daresbury Laboratory, England). In this preliminary analysis, resolution was limited to 4.8 Å.

Results and discussion

Free sialic acid is known to inhibit hemagglutination (Burns, Kenimer & Manclark, 1987). A survey of other simple saccharides showed inhibition only at concentrations above 500 m*M* for sucrose and galactose. Commercial preparations of NeuNAc at concentrations of 10 m*M* and above caused lysis of erythrocytes and inhibition of agglutination of the resulting ghosts. The mercuric analog, NeuNAcHg, inhibited agglutination at 1 m*M* concentrations, and was therefore a measurably stronger competitor than native NeuNAc for the saccharide binding site of PT. This may be because the commercial preparations are mixtures of α- and β-anomers (NMR results not shown) while NeuNAcHg is a pure α-anomer (Fig. 1).

Integrated intensity data from PT crystals soaked with NeuNAcHg resulted in a difference Patterson map which when analyzed yielded three obvious mercury sites (Fig.

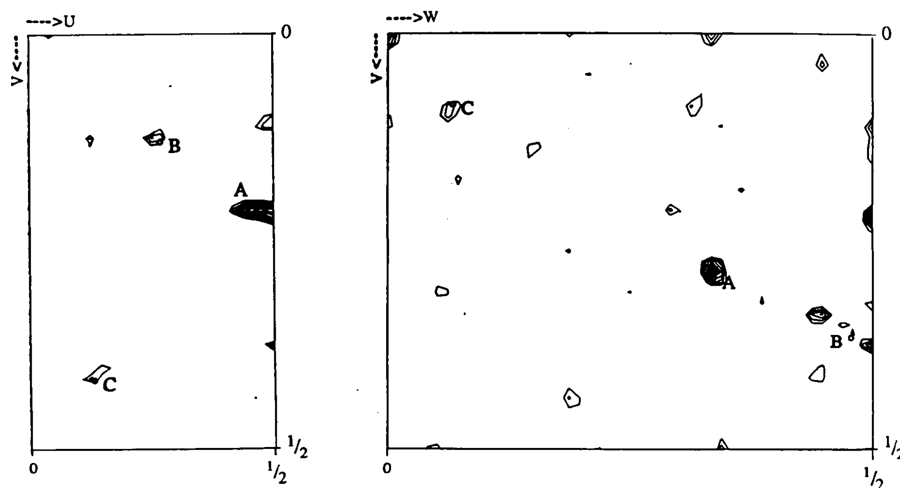

Fig. 2. W = 1/2 and U = 1/2 Harker sections of the NeuNAcHg difference Patterson show the three refined heavy-atom sites, labelled *A*, *B* and *C*. The plot is contoured by standard deviations, beginning at three standard deviations.

Table 1. *Statistics for native and derivative X-ray data*

$R_{sym} = \sum_h \sum_i |\langle I_h \rangle - I_{hi}| / \sum_h \sum_i I_{hi}$. $\langle I_h \rangle$ is the average measurement of reflection *h*. I_{hi} is the *i*th measurement of reflection *h*. The phasing power is $\langle f_c \rangle / \langle E \rangle$ where $\langle f_c \rangle$ is the mean amplitude of the heavy-atom model. $\langle E \rangle$ is the mean lack of closure as defined in Blundell & Johnson (1976, p. 356). FOM is the figure of merit as defined by Blow & Crick (1959).

Data set	Native	NeuNAcHg
X-ray wavelength (Å)	1.07	1.54
No. of crystals	3	1
No. of reflections	53974	54383
No. of unique reflections	12132	18683
R_{sym} (%)	7.8	6.1
Resolution limit (Å)	15 4.8	∞–4.1
% Complete	80.3	75.1
Mean $\Delta F/F$ (%)		16.6
Phasing power to 4.8 Å		1.42
FOM		0.504

2) in which all nine Harker peaks and 11 of 12 cross peaks were identified. The positions of the mercury sites refined well using the *REFINE*2 program from the *CCP*4 software suite (Table 1).

Isomorphous binding of the metallosaccharide is essential for the successful phasing of diffraction data collected from PT crystals. In the case of another AB_5 toxin, heat-labile enterotoxin (Sixma *et al.*, 1992), sugar binding in crystals induced structural changes sufficiently large that the method used here for phasing could not work due to lack of isomorphism (Crick & Magdoff, 1956) . In the case of PT crystals however, the interpretable Patterson map suggests that NeuNAcHg binding *per se* does not lead to conformational changes.

Protein structures the size of PT have been solved using a single derivative combined with non-crystallographic symmetry averaging and solvent flattening was used (Wilson, Skehel & Wiley, 1981). The non-crystallographic symmetry operators which arise from packing in PT crystals are not yet known. The SIR electron-density map from the single NeuNAcHg derivative is insufficient for solvent flattening and interpretation. Work is currently underway to identify other derivatives using phases from the NeuNAc heavy-atom model by cross difference Fourier techniques.

The synthetic strategy used for NeuNAcHg has been applied to galactose and fucose (unpublished results) and should be generally applicable to the preparation of mercuric β-saccharides or *N*-acetyl saccharides for phasing the X-ray structures of other carbohydrate-binding proteins.

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