ACTIVATION OF *VIBRIO CHOLERAE* NEURAMINIDASE BY DIVALENT CATIONS

Leif HOLMQUIST
National Defence Research Institute, Department 4, S–172 04 Sundbyberg 4, Sweden

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1. Introduction

Previous studies on *Vibrio cholerae* neuraminidase [1,2] have demonstrated that enzyme is activated by Ca^{2+}, Mn^{2+}, Co^{2+} and to a lesser extent by Mg^{2+}. After treatment with ethylene diamine tetraacetate (EDTA) and dialysis, the enzyme was found to lose its activity completely. Divalent metal ions like Hg^{2+} and Cu^{2+} which irreversibly inhibit enzymes containing sulfhydryl groups essential for activity are also reported to inactivate *Vibrio cholerae* and influenza virus neuraminidase respectively [2–4]. In contrast to these findings, it was observed that p-chloromercuribenzoate and other sulfhydryl group blocking reagents only slightly influenced the neuraminidase activity [5]. The stabilization of purified enzyme preparations by cyanide anions, was attributed to the complexing of heavy metal ions [1]. Convincing evidence has been presented that the carboxyl group in the nonulosaminic part of the neuraminidase substrate must bind to the enzyme in order to effect enzymatic cleavage of the α-ketosidic linkage [6,7]. As the divalent cations presumably in conjunction with the carboxyl group in the substrate may be essential for the formation of the enzyme-substrate complex [3] a study of the inhibition effects of various deactivating cations was undertaken. The present communication reports the effect of Fe^{3+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Ag^{+}, Li^{+}, Na^{+}, K^{+}, imidazolonium and ethylene diammonium ions on *Vibrio cholerae* neuraminidase partially activated by Ca^{2+}. In addition the effect of Ca^{2+} on the interaction of the neuraminidase with the recently described *N*-acetylneuraminic acid-Sepharose [8] in which the nonulosaminic acid is bound to the matrix by means of an α-ketosidic linkage, has been studied.

2. Materials and methods

2.1. *General methods*

Neuraminidase from *Vibrio cholerae* (glycoprotein *N*-acetylneuraminyl–hydrolase, EC 3.2.1.18) was purchased from Behring-Werke, Marburg; 1 ml containing 500 units (producer's specification). The enzyme was dialyzed against a 50 fold volume of 0.1 M chloride-free Tris–maleate buffer, pH 6.40 for 2 to 3 days at +4°C with frequent change of buffer. Benzyl α-ketoside of *N*-acetylneuraminic acid [9], twice recrystallized from anhydrous methanol was used as substrate and the cations examined were assayed as calcium chloride dihydrate or calcium nitrate tetrahydrate, copper (II) nitrate trihydrate, iron (II) sulfate heptahydrate, iron (III) chloride hexahydrate, mercury (II) nitrate, silver acetate, lithium chloride and sodium chloride respectively. Sepharose-bound 2-aminoethylaminocarbonylmetyl α-ketoside of *N*-acetylneuraminic acid type V was prepared as previously described [8].

2.2. *Enzyme assays*

Incubations with neuraminidase (12.5 units) were performed in 0.05 M Tris–maleate buffer pH 6.40, in a total volume of 0.5 ml at 37°C. The final concentration of benzyl α-ketoside of *N*-acetylneuraminic acid was 4 mM and of cations 0 to 2 mM. The pH of all solutions was carefully controlled and adjusted to 6.40. In experiments with Ag^{+}, chloride-free solutions and buffer were used. The enzyme assays were carried out by mixing 0.1 M Tris–maleate buffer pH 6.40 (250 μl), a solution of the cation in water (100 μl), neuraminidase (25 μl, 12.5 units) and water to make up the desired volume. After 5 min preincubation at 37°C,

a pH adjusted solution of the substrate in water (100 μl) was added. Samples (100 μl) were removed from the incubation mixture and immediately frozen at −70°C. Released *N*-acetylneuraminic acid was determined according to Warren [10]. A control experiment without added enzyme was also run. The tested cations did not affect the determination according to [10].

2.3. *Experiments with N-acetylneuraminic acid–Sepharose*

A column of *N*-acetylneuraminic acid–Sepharose (5 x 5 mm) was equilibrated with the appropriate buffer at 0°C. Dialyzed neuraminidase (0.3 ml, 150 units) was applied to the column and was allowed to penetrate into the gel. After 15 min, two 0.1 ml portions of buffer were added to the column and the enzyme eluted with 0.1 M Tris–maleate buffer pH 6.40 or 0.1 M sodium acetate buffer pH 5.50, without and with Ca^{2+} present in a 0.01 M concentration. Fractions of 2 ml were collected and a flow rate of 0.2–0.3 ml/min was used. To each fraction was added 0.2 M calcium chloride dihydrate in water (0.1 ml) or water (0.1 ml) in experiments with Ca^{2+} present in the eluant and a buffered solution of benzyl α-ketoside of *N*-acetylneuroaminic acid as substrate (800 μg) in water (50 μl). After incubation at 37°C for 30 min samples (200 μl) were removed from each fraction and analyzed according to Warren [10]. Desorption of neuraminidase from the columns was performed by addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the buffer (400 μg/ml). The effluent was treated as above omitting the addition of substrate.

3. Results and discussion

Numerous examples may be found of the specific binding of Hg^{2+}, Cu^{2+} and Ag^{+} to sulfhydryl groups in enzymes, frequently accompanied by loss of enzymatic activity. At increasing metal ion concentrations, primary amino groups, imidazole and carboxyl groups appear to coordinate with the metals [11].

In the present investigation on *Vibrio cholerae* neuraminidase, partially activated by Ca^{2+} at a 0.2 mM concentration, it was found that Cu^{2+} and Fe^{2+} induced enzyme activation. The use of a partially activated enzyme, assured its proper function and an activity proportional to the metal ion concentration. In accordance with previous findings [1], full enzymatic activity was restored by Ca^{2+} at a 1 mM concentration. The activating efficiency of the metals at pH 6.40, (the pH optimum of the enzyme) decreased in the order $Ca^{2+} > Cu^{2+} > Fe^{2+}$. Imidazolonium, ethylene diammonium, Fe^{3+}, Hg^{2+}, Ag^{+}, Li^{+}, Na^{+} and K^{+} when present in the incubation mixture at a 2 mM concentration produced no inhibition or stimulation of the partially activated neuraminidase, using the benzyl α-ketoside of *N*-acetylneuraminic acid as substrate. (fig. 1). The fact that neuraminidase was not inhibited by Ag^{+}, and Hg^{2+} and activating by Cu^{2+} and Fe^{2+} suggests that free sulfhydryl or primary amino groups important for catalytic activity are absent. The affinity of the enzyme for its substrate in the presence and absence of activating metal ions was examined, by means of Sepharose-bound 2-aminoethylaminocarbonylmethyl α-ketoside of *N*-acetylneuraminic acid (*N*-acetylneuraminic acid–Sepharose) [8].

Fig. 2 shows that a slightly increased leakage of enzyme could be detected when the column was eluted

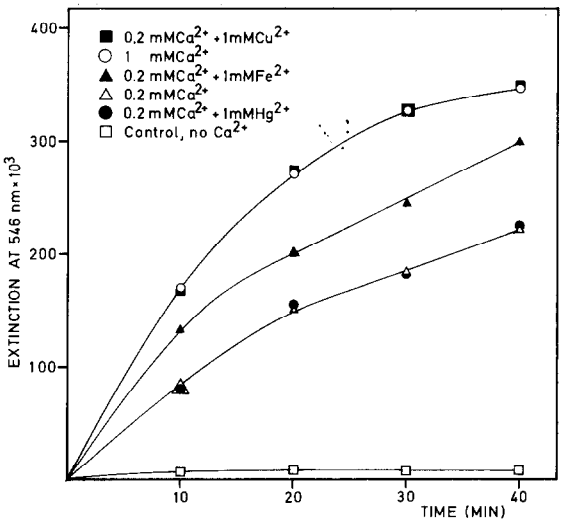

Fig.1. Activity of *Vibrio cholerae* neuraminidase at pH 6.40 in the presence of different divalent metal ions and with the benzyl α-ketoside of *N*-acetylneuraminic acid as substrate. Released *N*-acetylneuraminic acid is expressed as the intensity at 546 nm of the colour obtained with the thiobarbituric acid method according to [10].

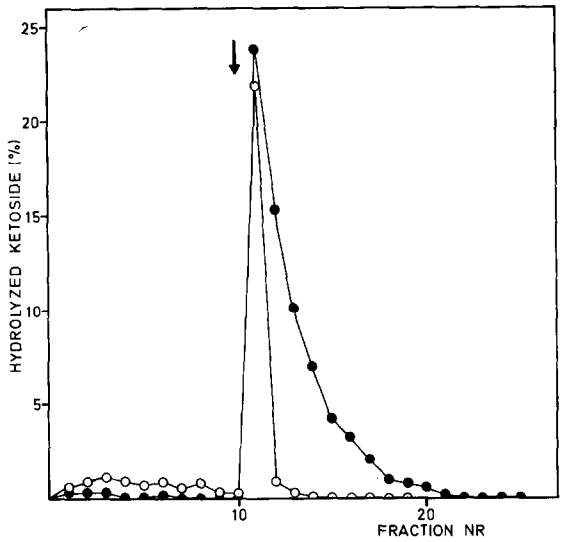

Fig.2. Activity of *Vibrio cholerae* neuraminidase in the effluent from a column of *N*-acetylneuraminic acid–Sepharose. Eluant: 0.1 M Tris–maleate buffer pH 6.40 with (•–•–•) and without Ca^{2+} (o–o–o). The arrow denotes the start of the addition of the benzyl α-ketoside of *N*-acetylneuraminic acid used to desorb the enzyme from the column. For further details see text.

with Ca^{2+}-free buffer, pH 6.40, compared with the results obtained in experiments with Ca^{2+} in the system. The addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluant resulted in an effective desorption of enzyme both in the absence and presence of Ca^{2+}.

However, in the absence of Ca^{2+} enzyme activity was lost. If the divalent metal mainly functions as an agent for the binding of the enzyme to the carboxyl group in the substrate, the enzyme should be adsorbed on a matrix-bound α-ketoside of *N*-acetylneuraminic acid, much more strongly in the presence of than in the absence of Ca^{2+} ions. It has previously been demonstrated [8] that the 2-hydroxyethyl α-ketoside of *N*-acetylneuraminic acid when coupled to Sepharose via an amide linkage (*N*-acetylneuraminamide–Sepharose) did not significantly adsorb the neuraminidase. Thus the present results suggest that the metal ions activating *Vibrio cholerae* neuraminidase are involved in the stabilization of the enzyme or an enzyme-substrate complex rather that in the direct binding of substrate to enzyme.

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