# THE PROTECTIVE ROLE OF PYRUVATE AGAINST HEAT INACTIVATION OF *N*-ACETYLNEURAMINATE LYASE

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## 1. Introduction

*N*-Acetylneuraminate lyase (EC 4.1.3.3) is the enzyme which catalyzes the reversible cleavage or synthesis of *N*-acetylneuraminic acid from pyruvate and *N*-acetyl-D-mannosamine [1]. It is widely distributed in animal tissues and in bacteria [2]. It consists of 2 subunits with a monomer $M_r$ ~50 000 and it has 2 active sites/native enzyme molecule [3]. The enzyme from *Clostridium perfrigens* belongs to the group of class I aldolases because it forms a Schiff base between the substrate pyruvate and a lysine residue of its active site in the course of the catalytic reaction [3–6].

It is known that most enzymes are quickly inactivated above 55°C. However binding of small molecules or ions can alter significantly the thermal stability of some proteins. A number of enzymes have been reported to be stable at high temperatures after interaction with substrates or coenzymes [7,8].

Here, we have studied the changes in the catalytic activity of *N*-acetylneuraminate lyase as a function of temperature in presence or absence of its substrates. Our results indicate that *N*-acetylneuraminate lyase is effectively protected against heat inactivation by pyruvate. The protective effect of pyruvate can be used in a new simple step of the purification procedure of the enzyme.

## 2. Materials and methods

*Clostridium perfrigens* (C-2638), *N*-acetylneuraminic acid, pyruvic acid and *N*-acetyl-D-mannosamine were purchased from Sigma. All other compounds and reagents used were of the highest commercially available purity.

*N*-Acetylneuraminate lyase (EC 4.1.3.3) was prepared from *Clostridium perfrigens* as in [9]. Activity assays of the enzyme were carried out to the direction of synthesis of *N*-acetylneuraminic acid from pyruvate and *N*-acetyl-D-mannosamine. The reaction mixture contained 10 μg enzyme/ml, 40 mM pyruvate, 40 mM *N*-acetyl-D-mannosamine and 100 mM potassium phosphate buffer (pH 7.2) in 0.2 ml final vol. and the assay was performed at 37°C. The *N*-acetylneuraminic acid formed was estimated as in [10]. For the kinetics of heat inactivation, samples of *N*-acetylneuraminate lyase (0.1 ml) containing 1 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.2) were preincubated at the desired temperatures. At various time intervals of the preincubation, aliquots were withdrawn, diluted 10-fold in potassium phosphate buffer (pH 7.2) and placed into ice-cold test tubes. The tubes were left on ice until all aliquots were collected. The activity of the enzyme was measured at 37°C as above.

Polyacrylamide gel electrophoresis was performed on a Shandon apparatus according to [11]. Protein was determined as in [12].

## 3. Results and discussion

Thermostability kinetic studies were performed after preincubation of purified *N*-acetylneuraminate lyase for 30 min at 37, 50, 60, 70, 80 and 100°C. As shown in fig.1, while the activity of the enzyme remains stable during its preincubation for 30 min at 37°C, preincubation at higher temperatures results to a gradual inactivation of the enzyme. Thus, it was found that after preincubation at 50°C and 60°C for 30 min the enzyme loses 15% and 55% of its original

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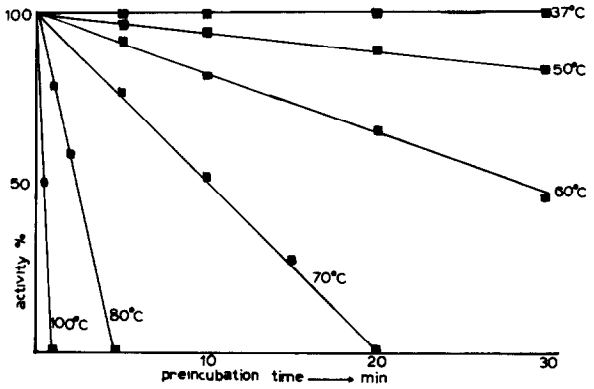


Fig.1. Rates of thermal inactivation of *N*-acetylneuraminate lyase at different temperatures. Enzyme activity was plotted as % of original activity as a function of preincubation time at 37, 50, 60, 70, 80 and 100°C. Original activity was taken as the activity of the enzyme at 37°C.

activity, respectively, while 10 min at 70°C results to an inactivation of 50%. The enzyme is completely inactivated after preincubation for 5 min at 80°C or for 2 min at 100°C. These findings suggest that *N*-acetylneuraminate lyase belongs to the wide category of non-thermostable enzymes.

Heat inactivation studies of *N*-acetylneuraminate lyase were also performed in the presence of its substrates, pyruvate and *N*-acetyl-D-mannosamine. It can be seen in fig.2, that the enzyme retained full activity

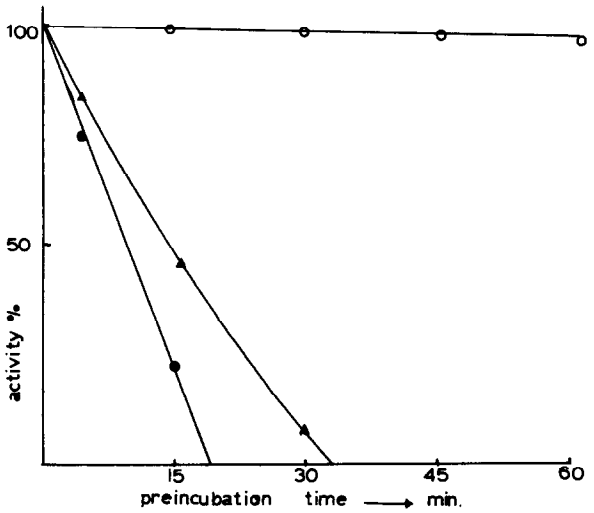


Fig.2. Rates of thermal inactivation of *N*-acetylneuraminate lyase at 70°C, in the absence (●) or in presence of 80 mM pyruvate (○) and 80 mM *N*-acetyl-D-mannosamine (▲). Enzyme activity was plotted as % of original activity as a function of preincubation time. Original activity was taken as the activity of the enzyme at 37°C.

after 1 h incubation at 70°C in the presence of 80 mM pyruvate. In contrast, the presence of 80 mM *N*-acetyl-D-mannosamine protects slightly the enzyme. As it was expected from these experiments the enzyme is completely inactivated during incubation for 1 h at 70°C.

In an attempt to elucidate the mechanism of enzyme protection against heat inactivation by pyruvate, the effect of high temperatures on the enzyme inactivation was studied in the presence of compounds which present structural analogies to pyruvate such as acetone, lactic acid, oxamic acid and α-ketoglutaric acid or pyridoxal phosphate which as it is well known forms a Schiff base with the ε-amino group of a lysine residue in the catalytic site of pyridoxal phosphate enzymes [13]. This property of pyridoxal phosphate is analogous to that of pyruvate which also forms a Schiff base with a lysine residue at the active site of *N*-acetylneuraminate lyase [5]. As can be seen in fig.3, from all compounds tested only-pyridoxal phosphate protects the enzyme substantially. These findings suggest that Schiff base formation between pyruvate and the ε-amino group of lysine at the active site of *N*-acetylneuraminate lyase might play a critical role in enzyme protection against heat inactivation. In addition it appears that the methyl- and carboxyl-groups

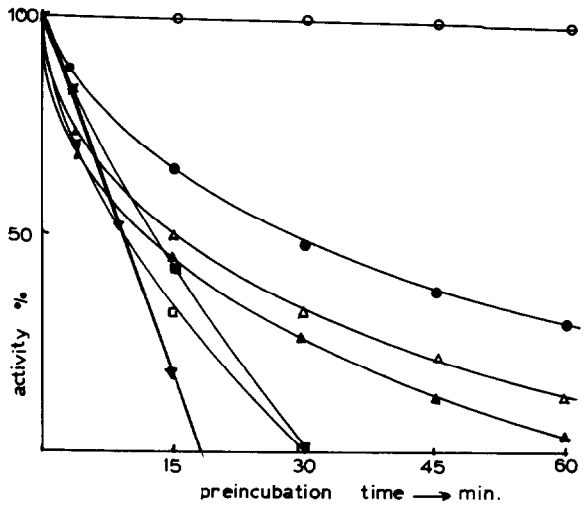


Fig.3. Rates of thermal inactivation of *N*-acetylneuraminate lyase at 70°C in the absence or in presence of structural analogues of pyruvate and pyridoxal phosphate. Pyruvate (○), α-ketoglutaric acid (△), lactic acid (▲), oxamic acid (■), acetone (□), pyridoxal phosphate (●), none (▼). All compounds were used at 80 mM final conc. except pyridoxal phosphate (2 mM). Enzyme activity was plotted as % of original activity as a function of preincubation time.

and the size of the pyruvate molecule seem to be indispensable for this protective effect. It is not known at present whether enzyme protection by pyruvate is due to the stabilization of heat-labile groups at the active site, prevention of thermal disruption of the enzyme molecule or stabilization of a particular conformation of the enzyme [14]. Additional work is needed for the elucidation of the mechanism of protection.

The protective property of pyruvate was further tested in respect to the purification procedure of the enzyme. An enzyme preparation after ammonium sulphate fractionation [9] was treated for 1 h at 70°C in presence of 80 mM pyruvate. The turbid solution was centrifuged and the supernatant was tested for *N*-acetylneuraminate lyase activity. A 10-fold increase in the specific activity of the enzyme (determined as in [15]) was succeeded by this single step in the purification procedure. In parallel experiments, the protein composition of the enzyme solutions before and after heat treatment in presence of pyruvate, was examined by polyacrylamide gel electrophoresis. As can be seen in fig.4, during the heating process a number of proteins were denatured and precipitated during centrifugation, while *N*-acetylneuraminate lyase remained active in the solution. These findings suggest that the protective property of pyruvate against heat inactivation of *N*-acetylneuraminate lyase can be used successfully for a simple procedure of the enzyme purification.

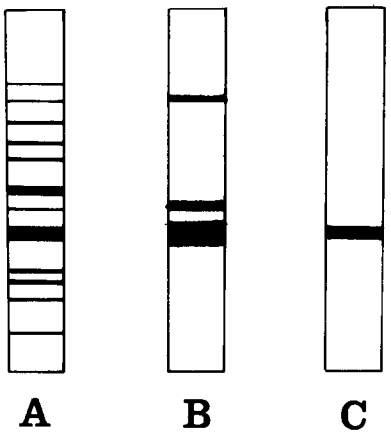


Fig.4. A schematic presentation of the results of polyacrylamide gel electrophoresis of a *N*-acetylneuraminate lyase ammonium sulphate preparation before (A) and after (B) thermal treatment, as compared to a purified enzyme preparation (C). The amount of protein applied to each gel was 50 μg for A and B and 10 μg for C.

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