# 1 Introduction

## 1.1 Biological Relevance of Sialic Acid and Neu5Ac Lyase

Sialic acids are a family of 2–keto–3–deoxy–5–amino–nonulopyranosonic sugar acids. Most lower eucaryotes, plants and most procaryotes do not appear to synthesise sialic acids. *N*–acetylneuraminic acid (sialic acid, Neu5Ac) is one common sialic acid frequently found as a terminal nonreducing sugar in complex glycoproteins and glycolipids of higher eucaryotes (Vimr & Troy, 1985a) and in gangliosides and some glycoproteins they also occur in the side position of oligosaccharides (Schauer, 1985). Sialic acid plays an important role in molecular recognition and it can control biological functions as diverse as viral infection and cellular adhesion. Because of its negatively charged nature, sialic acid is involved in binding and transport of positively charged compounds and in aggregation or disaggregation of cells (Schauer, 1985). Sialic acid found on the cell surface and on soluble glycoproteins regulates biological functions such as masking of sugar residues on oligosaccharides from specific receptors on cell surfaces (Ashwell & Morell, 1974; Schauer, 1985), modification of immune responses where sialic acid is an essential component of receptors (Woodruff & Gesner, 1969) and modulation of complement activation (Wedgwood *et al.*, 1956). Sialic acid influences the conformation of glycoproteins important in maintenance of the glycoprotein enzyme activity and in resistance of glycoproteins to proteases. In mammalian and microbial systems the *N*– and *O*–acylated sialic acids are antigenic determinants. Sialic acid has been found to prevent recognition of receptors by the corresponding ligands (Schauer, 1985).

*Escherichia coli* efficiently uses sialic acid as a sole carbon source which is dependent on induction of a sialic–acid specific transport activity (where sialic acid is transported without covalent modification) and an activity specific for cleaving sialic acids differentiable by their types of *N*– and *O*– acylations. Mutations affecting either transport or degradation of sialic acid prevent its use as a carbon source. Sialic acid is toxic to *E. coli* (Vimr & Troy, 1985a) either as the free sugar or in its activated cytidine 5'–monophospho– form (Vimr & Troy, 1985b) if accumulated intracellularly.

*N*–acetylneuraminate lyase (EC 4.1.3.3) is a specific enzyme for the degradation of non–glycosidically bound sialic acids into pyruvic acid and acylmannosamines (Schauer *et al.*, 1987) and catalyses the cleavage of Neu5Ac to pyruvate and *N*–acetyl–D–mannosamine (Figure 1) in a reversible reaction. The enzyme cleaves acylneuraminates having various *N*– and *O*– substituents with great difference in rates (Schauer, 1971). Neu5Ac lyase has a high degree of specificity for sialic acids with an apparent $K_m$ of 3.3 mM $\pm$ 0.1 mM for cleavage of Neu5Ac or *N*–Glycolylneuraminic acid (Aisaka *et al.*, 1991) and a low substrate specificity with regard to the *N*–susbtituents of neuraminic acid (Uchida *et al.*, 1984).

Neu5Ac lyase plays a key role in the regulation of the sialic acid metabolism in *Escherichia coli* (Vimr & Troy, 1985b). In order to participate in sialoglycoconjugate synthesis (in procaryotic and eucaryotic cells) Neu5Ac is activated to its cytidine 5'–monophospho–from ($CMP$–Neu5Ac) and its concentration affects the rate at which sialoglycoconjugates are expressed (Vimr & Troy, 1985b). Neu5Ac lyase regulates the intracellular concentration of sialic acid and therefore the concentration of $CMP$–Neu5Ac by modulating the pool of free sialic acid (Vimr & Troy, 1985b). High levels of Neu5Ac lyase are required to escape sialic acid toxicity (Vimr & Troy, 1985a). Neu5Ac lyase participates in dissimilating and detoxifying sialic acid that accumulates intracellularly after being added exogenously to the medium and regulates the intracellular level of sialic acid produced biosynthetically, presumably to recycle sialic acid carbon and to prevent overproduction of $CMP$–Neu5Ac (Vimr & Troy, 1985b). This control is as effective for sialic acid of biosynthetic origin as it is for exogenous sialic acid which may accumulate inside the bacterial cell (Vimr & Troy, 1985a). Sialic acid is also synthesised in bacteria by $N$–acetylneuraminate synthetase, which condenses $N$–acetylmannosamine and phosphoenolpyruvate (Uchida *et al.*, 1984).

Neu5Ac lyase also catalyses the reverse aldol condensation reaction and has been used for large scale synthesis of sialic acid, and some of its derivatives, from pyruvate and $N$–acetyl–D–mannosamine (Lilley *et al.*, 1992). The enzyme activity is of interest because of the growing appreciation of the role of sialic acid in controlling biomolecular interactions, particularly at the surface of cells. An analogue of sialic acid is presently in early stages of development as an anti–influenza virus compound (Itzstein *et al.*, 1993). Inhibitors of Neu5Ac lyase have potential anti–bacterial activity, because of the enzymes detoxifying role of sialic acid accumulated in cells.

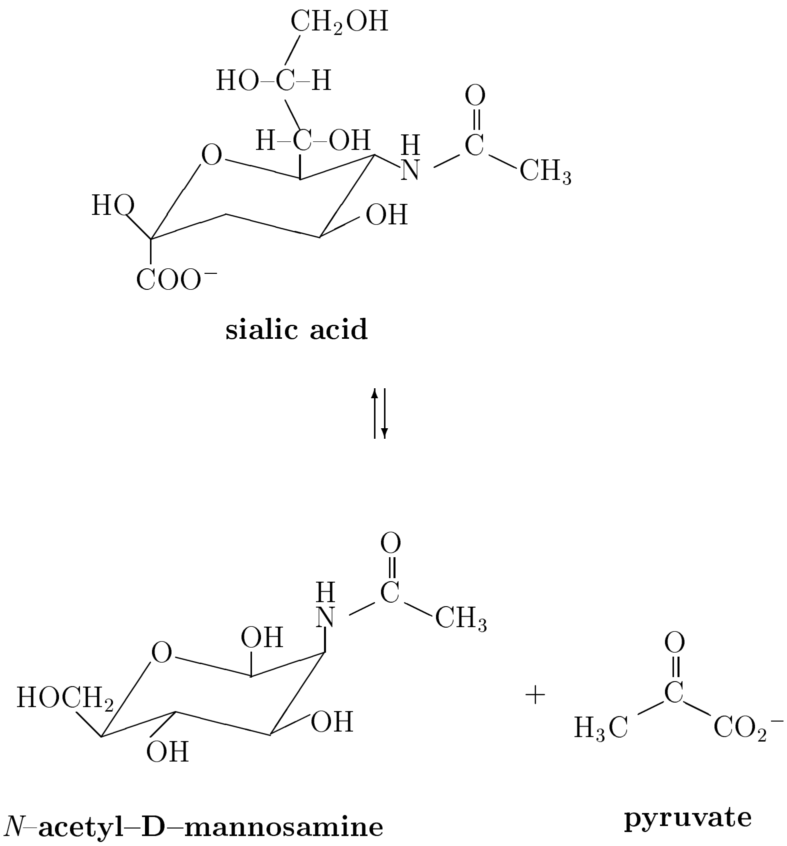


Figure 1: Cleavage of Neu5Ac (shown in its ring $\alpha$ configuration) by *N*–acetylneuraminate lyase. The enzyme also catalyses the reverse aldol condensation.

## 1.2 Biochemical Characterisation of Neu5Ac Lyase

Neu5Ac lyase is distributed in a wide variety of bacteria (Ohta *et al.*, 1986) and was originally found in some neuraminidase–producing pathogenic bacteria such as *Vibrio cholerae* and *Clostridium prefringens*; it has also been found in pathogenic bacteria such as *Cornybacterium diphtheriae* and *Pasteurella multocida* (Comb & Roseman, 1960; Arden *et al.*, 1972; Drzenick *et al.*, 1972) in some non–pathogenic bacteria such as *Escherichia coli* (Uchida *et al.*, 1984; Aisaka & Uwajima, 1986) and in mammalian tissues such as rat liver and brain, pig and bovine kidney (Comb & Roseman, 1960; Brunetti *et al.*, 1962; Sirbasku & Binkley, 1970). The gene encoding *E. coli* Neu5Ac lyase has been cloned and overexpressed (Lilley *et al.*, 1992; Ohta *et al.*, 1986; Aisaka *et al.*, 1991) and the enzyme has been biochemically characterised (Uchida *et al.*, 1984). Neu5Ac lyase is induced by free sialic acid (Vimr & Troy, 1985a; 1985b) and full enzyme induction requires relatively high concentrations of this sugar (>4 mM; Vimr & Troy, 1985b). The activity is regulated by product inhibition. Neu5Ac lyase is a typical inducible enzyme in micro–organisms that is produced only in the presence of its substrate Neu5Ac (Aisaka *et al.*, 1991). Cleavage and synthesis have a pH optimum around neutral (Uchida *et al.*, 1984; Deijl & Vliegenthart, 1983; Aisaka *et al.*, 1991). Chelating agents, alkylating reagents, $SH$–compounds and carbonyl group inhibitors are neither inhibitors nor activators of the enzyme (Uchida *et al.*, 1984; Aisaka *et al.*, 1991). Heavy metals such as $Ag^+$ and $Hg^{2+}$ are potent inhibitors (at 0.1 mM concentrations). $SH$–inhibitors such as $p$–chloromercuribenzoic acid and mercuric chloride and oxidising agents are highly inhibitory. One of the reaction products, pyruvate, inhibits the enzyme competitively at millimolar concentrations (70% inhibition at 10 mM) but *N*–acetyl–mannosamine does not (Uchida *et al.*, 1984; Aisaka *et al.*, 1991). The *E. coli* and *C. perfringens* enzyme have shown to be thermostable up to 60$^o$C and in the presence of pyruvate up to 75$^o$C (Uchida *et al.*, 1984; Aisaka *et al.*, 1991). The Neu5Ac lyase gene encodes a polypeptide of 297 amino acids (Ohta *et al.*, 1986) with a molecular weight of 32 kDa. Several reports have suggested that the enzyme is a trimer of these polypeptides in solution based on gel–filtration experiments (Uchida, *et al.*, 1984; Ohta *et al.*, 1986; Aisaka *et al.*, 1991).

## 1.3 Proposed Catalytic Residues

Intermediate Schiff's base formation have been established to be a general mechanism in reactions catalysed by aldolases (fructose 1,6–bisphosphate–aldolase, transaldolase, 2–oxo–3–deoxy–6–phosphogluconate aldolase, 2–deoxy–ribose–5–phosphate aldolase and 2–oxo–4–hydroxy–glutarate aldolase), where the Schiff's base forms between the carbonyl carbon $C^2$ (see Figure 1.1) of the substrate Neu5Ac (in the ring open form) and the $\epsilon$–amino group of a lysine residue of the active site of the enzyme molecule. This azomethine group ($>C=N$) can be reduced by sodium borotetrahydride. The borohydride reduction, leading to a stable covalent linkage of the substrate to the enzyme, is accompanied by a loss of enzyme activity. This irreversible inactivation of Neu5Ac lyase by borohydride occurs only in the presence of substrate and indicates that, in analogy to other aldolases, a Schiff's base between the substrate and the enzyme has been formed. Cyanide is known to be bound to double bonds of Schiff's bases forming a nitrile derivative. Suppresion of the enzyme activity by cyanide occurs only in the presence of substrate (Schauer, 1971).

Photo–oxidation of Neu5Ac lyase by illumination in the presence of Rose Bengal as a sensitiser rapidly and irreversibly inactivated the enzyme suggesting that histidine residue(s) are essential for activity. It has been suggested that the unprotonated imidazole nitrogen atom of a histidine with a pK of about 6.4 accepts the proton from the hydroxyl group $C^4$ (see Figure 1.1; Barnett *et al.*, 1971; Nees *et al.*, 1976). Histidine residues are involved in the enzyme reaction as base catalysis accepting a proton from $C^4$ hydroxyl group of the substrate and therefore facilitating the dealdolisation reaction in rabbit muscle aldolase (Horecker, 1969; Brand *et al.*, 1969). The participation of histidine or another proton acceptor in the catalytic process of Neu5Ac lyase is also expected, because substitution of the hydrogen atom at the $C^4$ hydroxyl group of Neu5Ac by an acetyl group has shown to reduce the rate of cleavage of $N$–acetyl–4–$O$–acetylneuraminate appreciably. The photo–inactivation of Neu5Ac lyase with Rose Bengal follows first order kinetics, in which histidine residues have been shown to be specifically destroyed by photo–oxidation. Attack on other residues than histidine cannot be excluded in these experiments, although Rose Bengal is believed to destroy histidine residues with some preference. The photo–inactivation is pH dependent (pK about 7) arguing in favour of catalytically active histidine residues. The suppression of the enzyme activity at pH 5.0, which is known to couple with histidine and tyrosine, indicates the presence of catalytic histidine residues (and/or tyrosine residues). The pH dependence of the photo–oxidation was only observed with histidine and not with tryptophan, methionine or tyrosine, which are also susceptible in photo–oxidation. Other proton acceptors than histidine cannot be ruled out (Schauer *et al.*, 1971).

Radioactive bromopyruvate was used to inactive the enzyme. The rapid rate of reaction of bromopyruvate with thiol compounds suggests that any free and available thiol group will react with bromopyruvate. Barnett *et al.* (1971) concluded that cysteine residues are probably at the enzyme active site and reacting with bromopyruvate. The inhibitory effect of PCMB on the activity supports the concept of $SH$ groups playing a role in the catalytic activity (Schauer *et al.*, 1971). The same can be expected for serine residues, as indicated by experiments with diisopropylfluorophosphate as inhibitor (Schauer *et al.*, 1971).

## 1.4 Proposed Reaction Mechanism

In analogy to other aldolases and according to the biochemical results, Schauer *et al.* (1987) proposed a possible reaction mechanism (Figure 1.1) involving a Schiff's base between the carbonyl group of Neu5Ac with the $NH_2$ from a lysine residue, where histidine serves as a proton acceptor. Barnett *et al.* (1971) proposed a possible cysteine residue being responsible for accepting the proton lost during the aldol condensation. The nucleophilic attack of the amino group of a lysine residue of the enzyme on $C^2$ of the substrate molecule (e.g. Neu5Ac) results in formation of a Schiff's base. The unprotonated imidazole nitrogen atom of a histidine residue serves as acceptor of the proton from the hydroxyl group at $C^4$ of neuraminic acid (Schauer *et al.*, 1987). The deprotonation leads to cleavage of the bond between $C^3$ and $C^4$ (Schauer *et al.*, 1987). This mechanism has been elucidated by photo–oxidation of lyase, reagents for histidine modification, and borohydride reduction in the presence of the substrate (Schauer *et al.*, 1987).

Although never detected, the open form of Neu5Ac is believed to be an intermediate in the $\alpha - \beta$ mutarotation in analogy to other sugars. In aqueous solution the $\beta$–anomer concentration is a factor of twenty larger than that of the $\alpha$–anomer. Deijl & Vliegenthart (1983) performed several experiments using sialidase to generate the substrate for aldolase. $\alpha$–Glycosides of Neu5Ac inhibit the aldolase action which has led to the suggestion that Neu5Ac binds to the enzyme in a configuration that greatly resembles the conformation of its $\alpha$–anomer. It is possible that the $\alpha$–anomer itself binds to the enzyme and

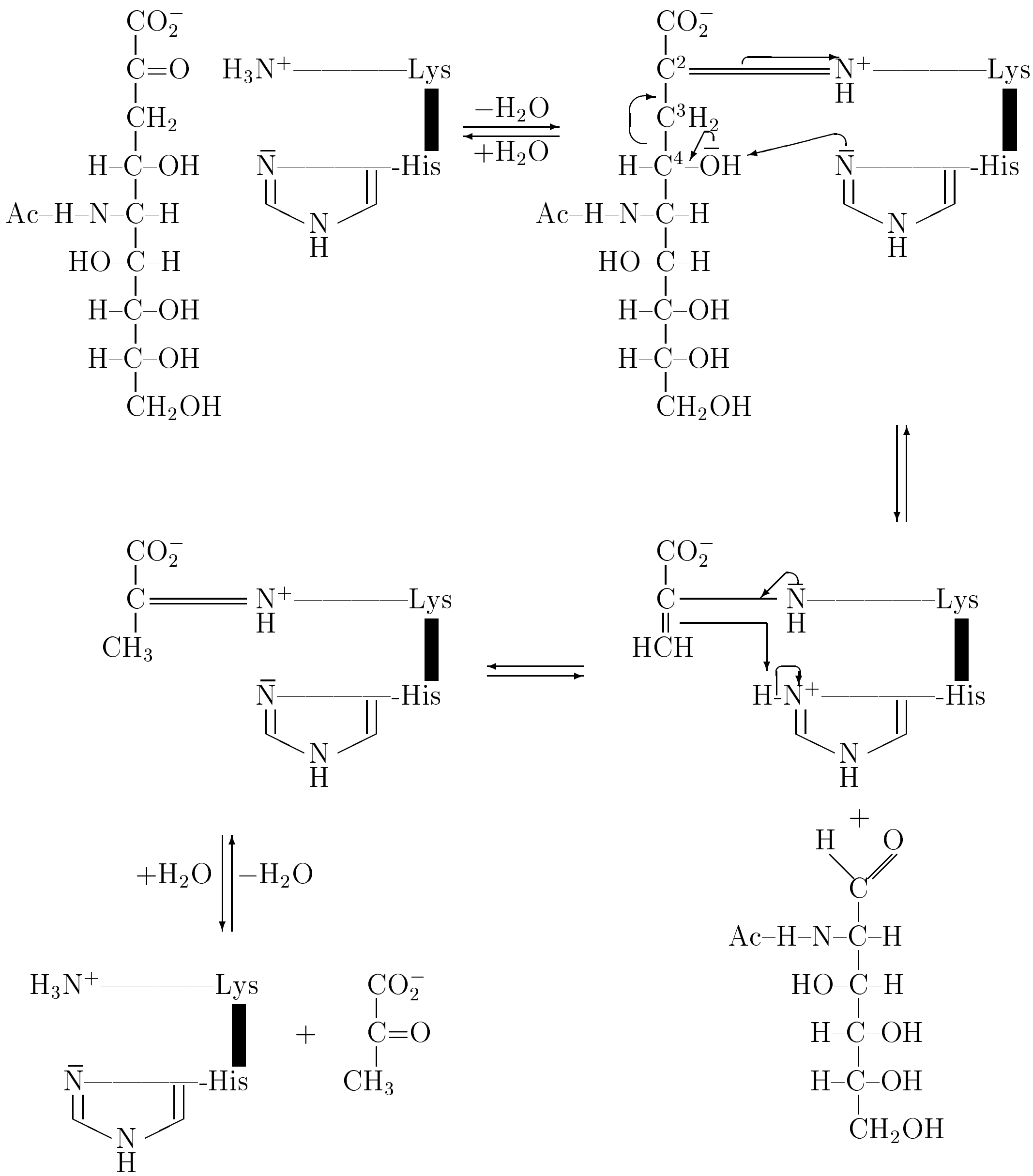

Figure 2: Reaction mechanism of the reversible cleavage of the substrate molecule (e.g. Neu5Ac; shown here in its open form) by Neu5Ac lyase proposed by Schauer *et al.* (1987). A covalent bond between $C^2$ of Neu5Ac and the $\epsilon$–amino nitrogen atom from a lysine residue in the enzyme is formed. After formation of this Schiff's base, the unprotonated imidazole nitrogen atom of a histidine residue or another proton acceptor serves as acceptor of the proton from the hydroxyl group at $C^4$ of neuraminic acid. This deprotonation leads to cleavage of the bond between $C^3$ and $C^4$.

undergoes ring opening at the active site. This is supported by the observation that the $\beta$–anomer does not serve as a substrate and its $\beta$–glycosides do not inhibit the enzymatic action. Deijl & Vliegenthart (1983) therefore concluded that the open chain form, allowing the formation of the intermediate Schiff's base during enzyme reaction between the carbonyl group of sialic acid with the $-NH_2$ of lysine, must strongly resemble that of the $\alpha$–anomer.

## 1.5 Overview

This work describes the crystallisation of Neu5Ac lyase from *E. coli* and presents its three–dimensional structure studied by Single Isomorphous Replacement with symmetry averaging and phase extension. Crystallographic refinement has been carried out to 2.2 Å resolution. The active site of the enzyme has been tentatively identified and possible catalytic residues are discussed. X–ray diffraction experiments with substrates into the crystal have been attempted in order to provide insight into the reaction mechanism and to confirm whether the proposed catalytic residues are correctly positioned to participate in catalysis. Further experiments are also suggested.

The three–dimensional structure of the enzyme is compared with that of other aldolases of known three–dimensional structure. Sequence homology between Neu5Ac lyase, *E. coli* dihydrodipicolinate synthase (DHDPS) and the *mosA* gene product of *Rhizobium meliloti* suggest that these proteins will have similar three–dimensional structures.