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Purification, crystallization and characterization of *N*-acetylneuraminate lyase from *Escherichia coli*

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N-Acetylneuraminate lyase produced by *Escherichia coli* was purified and crystallized from a genetically engineered strain (*E. coli* SF8/pNAL1). The enzyme showed apparent molecular masses of 105000 Da on gel filtration and 35000 Da on SDS/PAGE, suggesting that the enzyme is a trimer. The apparent optimum pH and temperature were found to be 6.5–7.0 and 80 °C respectively. The K_m values for *N*-acetylneuraminate and *N*-glycollylneuraminate were 3.3 and 3.3 mM respectively. The enzyme was inhibited by reduction with $NaBH_4$ in the presence of the substrate, indicating that the enzyme belongs to the Schiff-base-forming Class I aldolases. The enzyme was strongly inhibited by Cu^{2+} ions, *p*-chloromercuribenzoate and *N*-bromosuccinimide, and also inhibited competitively by the reaction product, pyruvate, and its structurally related compounds, dihydroxyacetone and DL-glyceraldehyde.

INTRODUCTION

N-Acetylneuraminate (AcNeu) lyase (EC 4.1.3.3) cleaves AcNeu to pyruvate and *N*-acetyl-D-mannosamine. This enzyme is one of the key enzymes in sialic acid metabolism [1], and has been found in mammalian tissues, in some pathogenic bacteria, such as *Clostridium perfringens*, *Corynebacterium diphtheriae*, *Pasteurella multocida* and *Vibrio cholerae* [2–5], and in some non-pathogenic bacteria, such as *Escherichia coli* [6,7]. Recently, Vimr & Troy reported that AcNeu lyase can efficiently modulate the intracellular concentration of sialic acid (AcNeu) [8,9].

AcNeu lyase is a typical inducible enzyme in micro-organisms that is produced only in the presence of the substrate (AcNeu) [6,7]. The high cost of AcNeu was a big obstacle to the production and detailed characterization of the enzyme. AcNeu lyase from *Cl. perfringens* has been relatively well investigated by Schauer *et al.* [10,11]. Purification of AcNeu lyase from *E. coli* and its enzymic properties have also been reported [12], though the enzyme has not been fully characterized.

We have succeeded in cloning and constitutively expressing the AcNeu lyase gene of *E. coli* [7], and have developed a method for large-scale production of the enzyme [13]. On the basis of those results, we intended to characterize the *E. coli* AcNeu lyase more fully. In the present paper, we describe the purification and enzymic properties of AcNeu lyase from the gene-cloned strain of *E. coli*.

MATERIALS AND METHODS

Materials

AcNeu was purchased from Nakarai Chemicals (Kyoto, Japan). DEAE-Sephadex (A-50), Sephadex G-150, Polybuffer exchanger (PBE94) and Polybuffer (74) were obtained from Pharmacia–LKB Biotechnology (Uppsala, Sweden). Hydroxyapatite was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Marker proteins for molecular-mass determination were purchased from Pharmacia–LKB Biotechnology and Oriental Yeasts Co. (Osaka, Japan). Other chemicals were all analytical-grade reagents.

Micro-organism and culture conditions

E. coli C600-SF8 harbouring recombinant plasmid (pNAL1) containing the AcNeu lyase gene [7] was used as a source of the enzyme for purification. The medium for cultivation consisted of 1.0% (w/v) peptone, 0.7% (w/v) meat extract, 0.3% (w/v) NaCl, pH 7.0, and 0.002% (w/v) ampicillin. Cultivation was carried out in a 30-litre jar fermenter containing 18 litres of the medium at 28 °C for 18 h with aeration (18 litres/min) and agitation (300 rev./min). The cells were harvested by continuous-flow centrifugation. The yield of wet cells was approx. 6 g/litres of medium.

Enzyme assay

AcNeu lyase activity was assayed by measuring the amount of pyruvate formed by using lactate dehydrogenase and NADH as described previously [7] (assay method I). This method was used throughout the purification procedures and for most investigations of the enzymic properties. In some inhibition studies, AcNeu lyase activity was assayed by measuring colorimetrically the amount of *N*-acetyl-D-mannosamine formed as described by Reissig *et al.* [14] (assay method II). Protein concentration was determined by the method of Lowry *et al.* [15]. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of pyruvate/min. Specific activity was expressed as units/mg of protein.

Electrophoresis in polyacrylamide gel

Native PAGE was performed in 10% (w/v) disc gels, based on the method of Davis [16]. SDS/PAGE was conducted by the method of King & Laemmli [17] using SDS (0.1%, w/v)/polyacrylamide (12.5%, w/v) gel (1 mm thick). The gels were stained for protein with Coomassie Brilliant Blue.

Ultracentrifugal analysis

Analytical ultracentrifugation was carried out with a Hitachi model UCA-1 ultracentrifuge at 20 °C, by the method of Vesterberg & Svensson [18].

N-Terminal amino acid sequencing

The *N*-terminal sequence of the purified enzyme was determined with an Applied Biosystems 470A gas-phase sequencer.

Molecular-mass determination

The molecular mass of the enzyme was estimated by gel filtration on a Sephadex G-150 column (2.5 cm × 50 cm) by the

Abbreviations used: AcNeu, *N*-acetylneuraminate; GcNeu, *N*-glycollylneuraminic acid; PEG, poly(ethylene glycol).

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method of Andrews [19], and by SDS/PAGE as described above. The molecular mass was determined from the relative elution volumes or mobilities of standard proteins.

RESULTS

Purification of AcNeu lyase from *E. coli* SF8/pNAL1

Purification was carried out at 0–5 °C, unless otherwise specified.

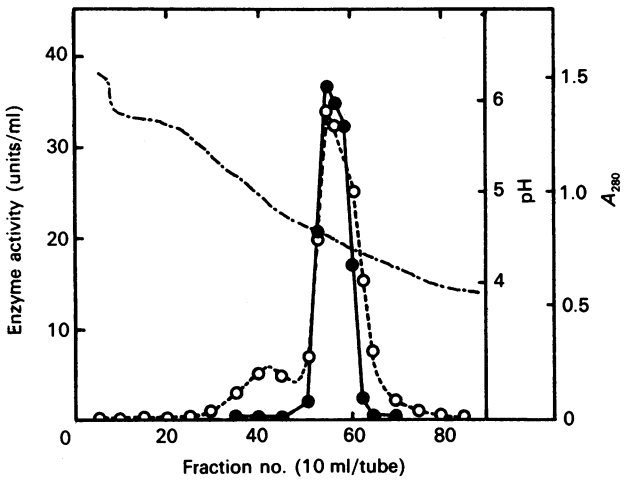

Fig. 1. Chromatofocusing of AcNeu lyase

The enzyme solution was applied to a Polybuffer exchanger (PBE94) column (2 cm × 7 cm) and eluted as described in the text. Fractions were assayed for AcNeu lyase activity (●), protein (A_{280}; ○), and pH (–·–·–).

Cell-free extract. *E. coli* SF8/pNAL1 cells (about 100 g wet wt.) were suspended in 1 litre of 10 mM-potassium phosphate buffer, pH 7.0, and homogenized in a Dyno-Mill (W. A. Bachofen, Basel, Switzerland). The homogenate was centrifuged at 12000 *g* for 30 min to remove cell debris, and the supernatant was collected as cell-free extract.

$(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation with continuous stirring. After 1 h, the precipitate formed was removed by centrifugation. The resultant supernatant was adjusted to 90% saturation with additional $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 12000 *g* for 30 min, dissolved in 200 ml of 10 mM-potassium phosphate buffer, pH 7.0, and dialysed against two 5-litre volumes of the same buffer.

Heat treatment. The dialysis residue was heated at 70 °C for 15 min. The resulting precipitates were removed by centrifugation at 12000 *g* for 30 min.

DEAE-Sephadex column chromatography. The supernatant was applied to a DEAE-Sephadex A-50 column (5 cm × 100 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, and the enzyme was eluted with a linear concentration gradient of 0–1.0 M-NaCl in the same buffer. Active fractions were pooled, concentrated to about 10 ml on an Amicon YM10 membrane, and dialysed against 2 litres of 10 mM-potassium phosphate buffer, pH 7.0, at 4 °C overnight.

Sephadex G-150 gel filtration. The dialysis residue (12 ml) was placed on the surface of a Sephadex G-150 column (5 cm × 100 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, and the proteins were eluted with the same buffer. Active fractions (12 ml) were pooled, and concentrated to 5 ml on a YM10 membrane.

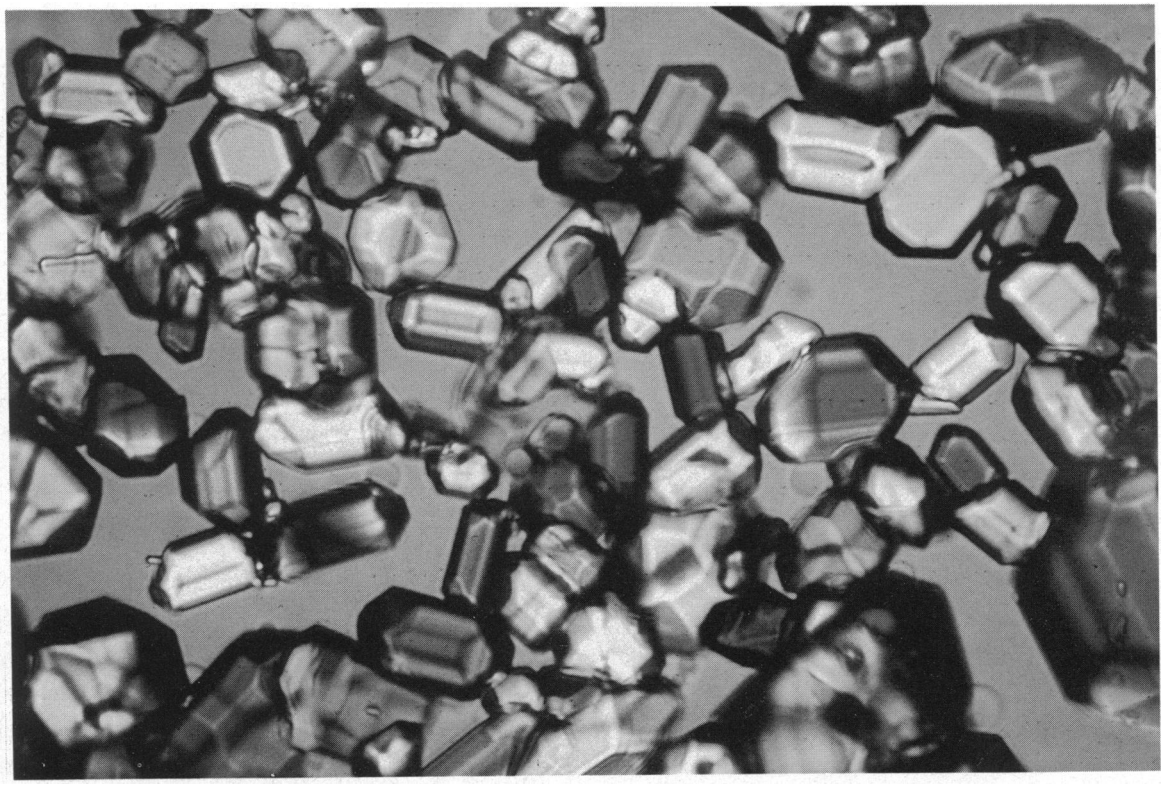

Fig. 2. Crystalline AcNeu lyase from *E. coli* SF8/pNAL1

Hydroxyapatite column chromatography. The concentrate (5 ml) was applied to a hydroxyapatite column (2 cm × 10 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. The column was washed with the same buffer, and the enzyme was eluted with 100 mM-potassium phosphate buffer, pH 7.0. Active fractions were pooled, concentrated to 5 ml on a YM10 membrane, and dialysed against 1 litre of 25 mM-histidine/HCl buffer, pH 6.2, at 4 °C overnight.

Chromatofocusing. The dialysis residue (6 ml) was applied to a polybuffer exchanger (PBE94) column (2 cm × 7 cm) equilibrated with 25 mM-histidine/HCl buffer, pH 6.2. The enzyme was eluted with Polybuffer 74 (200 ml) adjusted to pH 4.0 with HCl (Fig. 1). Active fractions were pooled, and the enzyme was precipitated with the addition of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 12000 ***g*** for 30 min, dissolved in 10 mM-potassium phosphate buffer, pH 7.0, and dialysed against the same buffer at 4 °C overnight.

Crystallization. Solid $(NH_4)_2SO_4$ was gradually added to the purified enzyme solution containing 30% PEG 6000 until it became slightly turbid; it was kept at 4 °C. Rhombohedral-shaped crystals appeared after 3 days (Fig. 2).

The procedures for the purification of AcNeu lyase are summarized in Table 1. The enzyme was purified about 90-fold from cell-free extract with 12% activity yield.

Properties of AcNeu lyase

Homogeneity. The purified enzyme preparation gave a single band on both native PAGE and SDS/PAGE, as shown in Figs. 3(*a*) and 3(*b*). The enzyme also showed a single and symmetrical moving peak on ultracentrifuge analysis, and the apparent sedimentation coefficient, $s_{20,w}$, was calculated to be 5.9 S (results not shown). These results indicate that the purified enzyme preparation was homogeneous.

***N*-Terminal amino acid sequence.** The amino acids at the *N*-terminus of the purified enzyme were sequenced, and 23 amino acid residues were determined. It lacked methionine at the *N*-terminus, and was Ala-Thr-Asn-Leu-Arg-Gly-Val-Met-Ala-Ala-Leu-Leu-Thr-Pro-Phe-Asp-Gln-Gln-Gln-Ala-Leu-Asp-Lys-. The sequence was in good agreement with the sequences of AcNeu lyases from other *E. coli* strains reported by Kawakami *et al.* [20] and Ohta *et al.* [21], except that the sequence reported by Ohta *et al.* contained methionine at the *N*-terminus. In our case the *N*-terminal methionine seemed to have been processed effectively by methionylaminopeptidase in spite of its increased production, because it was followed by a small amino acid, alanine, as described by Hirel *et al.* [22].

Table 1. Purification of AcNeu lyase from *E. coli* SF8/pNAL1

AcNeu lyase was purified from about 100 g of *E. coli* SF8/pNAL1 cells. Activities were determined by assay method I. Detailed conditions are described in the text.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1. Cell-free extract	65300	25300	0.387	100
2. $(NH_4)_2SO_4$ (50–90%)	25300	17500	0.692	69.2
3. Heat (70 °C, 15 min)	6900	17300	2.51	68.4
4. DEAE-Sephadex	752	16100	21.4	63.6
5. Sephadex G-150	461	13200	28.6	52.2
6. Hydroxyapatite	199	7010	35.2	27.7
7. Chromatofocusing (pH 6.2–4.0)	88.1	3130	35.5	12.4

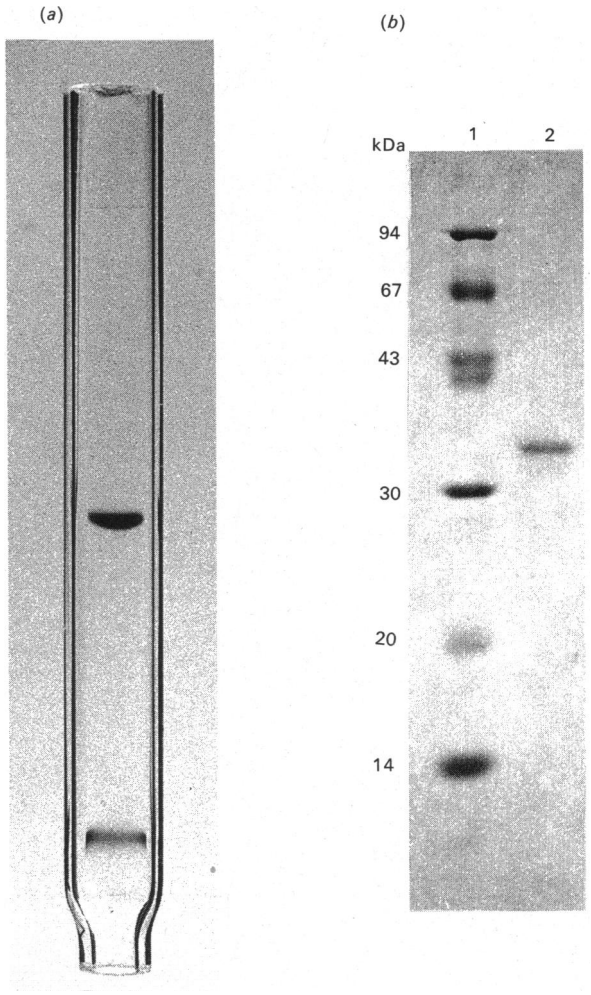

Fig. 3. Criteria for purity of AcNeu lyase

(*a*) Native PAGE pattern. Purified AcNeu lyase (6 μg) was subjected to native PAGE. (*b*) SDS/PAGE pattern. Track 1 contained molecular-mass marker proteins. Track 2 contained purified AcNeu lyase (4 μg). The conditions are given in the Materials and methods section.

Molecular mass. The apparent molecular mass of the purified enzyme was determined to be 105000 ± 10000 kDa (mean ± range of two estimations) by Sephadex G-150 gel filtration and 110000 ± 10000 kDa by h.p.l.c. with TSK gel G3000SW. On the other hand, analysis of the purified enzyme by SDS/PAGE revealed a single protein band, as shown in Fig. 3(*b*), with an apparent molecular mass of 35000 ± 2000 Da. These results suggest that the enzyme is composed of three identical subunits.

Isoelectric point. The pI of AcNeu lyase was estimated to be 4.5 ± 0.1 by chromatofocusing (Fig. 1) and isoelectric focusing (results not shown).

Effect of pH. To examine the pH optimum for the AcNeu lyase reaction, the enzyme reaction was carried out under the standard conditions except that the pH of the reaction mixture was varied. The enzyme was most active at pH 6.5–7.0. To examine the pH-

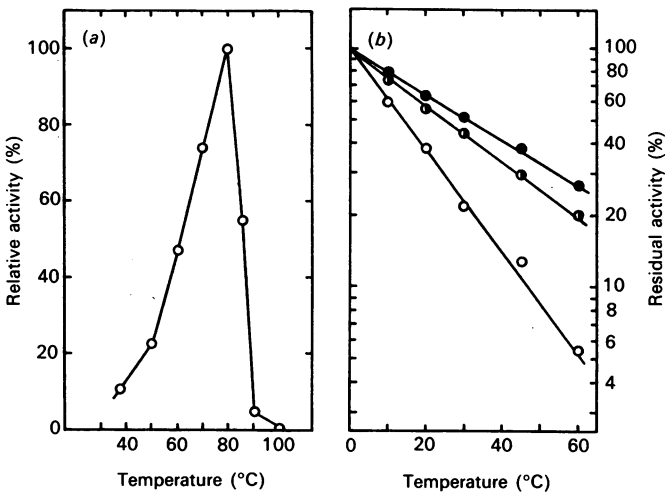

Fig. 4. Effect of temperature on the activity and stability of AcNeu lyase

(*a*) Reaction was carried out under the standard conditions (assay method I) at various temperatures as indicated. (*b*) The enzyme was kept at 85 °C in 30 mM-potassium phosphate buffer containing 5 mM-AcNeu (●), 5 mM-pyruvate (◑) or neither (○). At the intervals indicated, samples (5 μl) were withdrawn and their remaining activities were determined by assay method I.

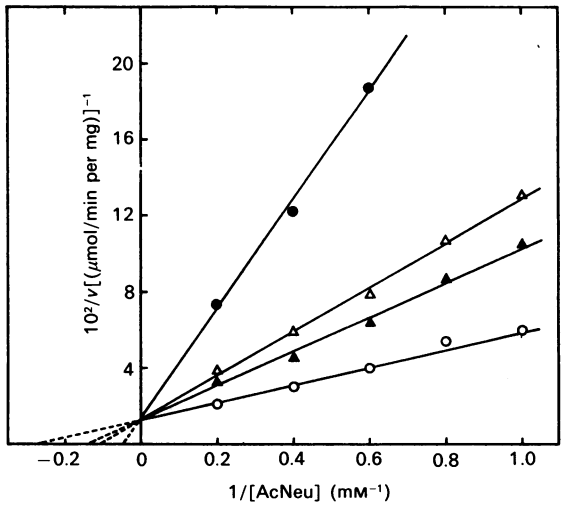

Fig. 5. Inhibition of AcNeu lyase by pyruvate and related compounds

The enzyme was preincubated in 62.5 mM-potassium phosphate buffer, pH 7.0, containing pyruvate, dihydroxyacetone or DL-glyceraldehyde at 25 °C for 15 min. Then the reaction was initiated by addition of AcNeu, and the initial velocity was determined by assay method II in the presence of (final concns.) 10 mM-pyruvate (●), 1 mM-dihydroxyacetone (▲), 1 mM-DL-glyceraldehyde (△) or no addition (○). Each point represents the mean of the nearest two of three similar experiments. K_i values were calculated from the equation $K_p = K_m(1+[i]/K_m)$, where K_m and K_p are apparent Michaelis–Menten constants in the absence and presence of an inhibitor respectively and [i] is the concentration of the inhibitor.

stability of the enzyme, it was left in 50 mM-potassium phosphate buffer of various pH values for 15 min at 75 °C, and the remaining activity was assayed under standard conditions. No appreciable loss of activity was found between pH 6.0 and 7.0. About 60% and 30% activity losses were observed at pH 5.0 and pH 8.0 respectively.

Effect of temperature. The effect of temperature on the enzyme activity was examined. The apparent optimum temperature for AcNeu lyase reaction (10 min) was about 80 °C (Fig. 4*a*). The activation energy of the enzyme reaction between 37 and 70 °C was estimated at approx. 51.9 kJ/mol (12.4 kcal/mol) from the Arrhenius plot. The thermal stability of the enzyme was examined. It was stable up to 70 °C for 15 min in 62.5 mM-potassium phosphate buffer, pH 7.0, and 50% inactivation was achieved at 82 °C. This thermal stability was further enhanced in the presence of AcNeu or pyruvate. That is, in the control, incubation for 15 min at 85 °C resulted in 52% inactivation of the enzyme, whereas in the presence of 5 mM-AcNeu or -pyruvate the inactivation was 28% or 34% respectively (Fig. 4*b*). A similar protective role of pyruvate against heat inactivation of AcNeu lyase has also been observed for the enzyme from *Cl. perfringens* [23].

Substrate specificity and reaction kinetics. The rates of cleavage of several sialic acids and related compounds by the purified enzyme were examined. *N*-Glycollylneuraminic acid (GcNeu) was cleaved at 20% of the rate of AcNeu. On the other hand, there was no detectable cleavage of colominic acid, αα′-2–8 homopolymer of AcNeu or 2-oxocarboxylic acids such as 2-oxohexanoic acid, 2-oxo-octanoic acid, 2-oxo-3-deoxyoctanoic acid and 2-oxononanoic acid. Thus AcNeu lyase showed a high degree of specificity for sialic acids.

The relationships between activity and substrate concentration were of the Michaelis–Menten type; the apparent K_m values for AcNeu and GcNeu were 3.3±0.1 (mean±range of two estimations) and 3.3±0.2 mM, and $V_{max.}$ values for AcNeu and GcNeu were 71.4±4.8 and 14.0±1.4 μmol/min per mg of protein respectively.

Inhibitors. The effect of various chemicals on the enzyme activity was examined. The enzyme was preincubated in 62.5 mM-potassium phosphate buffer, pH 7.0, containing various chemicals at 25 °C for 15 min. Then the reaction was initiated by addition of AcNeu. Of eight bivalent cations (1.0 mM each) tested, only $CuSO_4$ showed a strong inhibitory effect (93% inhibition). $FeSO_4$ had a slight inhibitory effect (20% inhibition), but other metal ions showed no appreciable effects. Chelating agents (EDTA, αα′-bipyridyl, 8-hydroxyquinoline, diethyldithiocarbamate; 1.0 mM each) did not inhibit the enzyme activity at all. Of the thiol-blocking reagents tested, *p*-chloromercuribenzoate, a mercaptide-forming reagent, showed a strong inhibitory effect (56% inhibition at 0.001 mM and 100% inhibition at 0.01 mM), but alkylating reagents (iodoacetate and iodoacetamide; 1.0 mM each) did not inhibit the enzyme activity. *N*-Bromosuccinimide, a relatively specific tryptophan-binding reagent, showed 64% inhibition at 0.01 mM and 100% inhibition at 0.1 mM, and diethyl pyrocarbonate, a relatively specific histidine-binding reagent [24,25], showed 72% inhibition at 1 mM.

The effect of reaction products and a variety of their related compounds on the enzyme activity was also examined. *N*-Acetyl-D-mannosamine and related compounds (D-mannosamine, *N*-acetyl-D-glucosamine) showed no appreciable effect at 10 mM, which is the same concentration as the substrate AcNeu. On the other hand, pyruvate showed a strong inhibitory effect (70% inhibition at 10 mM). Their double-reciprocal plots indicated competitive inhibition, with a K_i of 2.4±0.1 mM for AcNeu (Fig. 5). Dihydroxyacetone and DL-glyceraldehyde were more potent inhibitors (98% and 100% inhibition respectively at 10 mM) than pyruvate. These were also compatible with competitive inhibition with apparent K_i values of 1.1±0.1 mM and 0.67±0.04 mM for AcNeu respectively (Fig. 5). Methyl-

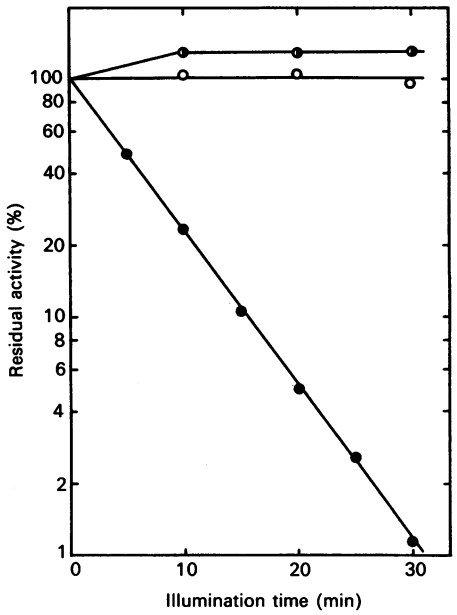

Fig. 6. Inactivation of AcNeu lyase by photo-oxidation

The enzyme was illuminated in 100 mM-potassium phosphate buffer, pH 7.0, containing 7.3 μM-Rose Bengal in a quartz cell at 10 °C with an incandescent lamp (60 W) at 10 cm (●). At intervals indicated, samples (100 μl) were withdrawn and remaining activities were determined by assay method I. The controls were incubated with Rose Bengal without illumination (○) or illuminated without Rose Bengal (◑).

Table 2. Inactivation of AcNeu lyase by $NaBH_4$

The enzyme was reduced at 0 °C with 100 mM-$NaBH_4$ in 250 mM-potassium phosphate buffer (pH 7.0) containing 2.5 mM compound where indicated. The solution during this reduction was maintained at pH 7.0 by the addition of 5 M-acetate. After 20 min at 0 °C, samples (200 μl) were withdrawn and their remaining activities were determined by assay method I.

Addition	Relative activity (%)
None	100
$NaBH_4$	59.7
$NaBH_4$ + AcNeu	5.2
$NaBH_4$ + pyruvate	6.2
$NaBH_4$ + *N*-acetyl-D-mannosamine	57.9

glyoxal and oxomalonate showed moderately inhibitory effects (50% and 42% inhibition respectively at 10 mM), but related compounds, such as DL-lactate, D-glycerate, oxamate, oxaloacetate and α-oxoglutarate, did not inhibit the enzyme activity significantly. Thus the structural characteristics of these inhibitors were as follows: (a) an oxo or aldehyde residue was indispensable; (b) C_3 compounds were inhibitory, but compounds containing more than three carbon atoms and containing nitrogen in place of carbon were not inhibitory.

Photo-oxidation. Photo-oxidation of AcNeu lyase was examined. Illumination of the enzyme in the presence of Rose Bengal as a sensitizer rapidly and irreversibly inactivated the enzyme. The loss of the enzyme activity followed first-order kinetics (Fig. 6), as has been observed in several other enzymes in which histidine residues have been shown to be specifically destroyed by photo-oxidation [10,25]. No loss of activity occurred when the enzyme was incubated with Rose Bengal in the dark or was illuminated without the sensitizer. The effect of pH of the enzyme solution on photoinactivation was also examined. Treatment at pH 6.0, 7.0 and 8.0 for 5 min caused approx. 64%, 61% and 50% losses respectively of the original activity. Thus the rate of photo-oxidation was relatively independent of pH. These results suggest that histidine residue(s) are essential for the activity of AcNeu lyase, as shown in other Class I aldolases [26].

Reduction with borohydride. To examine the involvement of Schiff-base formation in the AcNeu lyase reaction, the enzyme was treated with $NaBH_4$ in the presence or absence of substrate or product in accordance with the method of Grazi *et al.* [27]. As shown in Table 2, significant inactivation of AcNeu lyase by $NaBH_4$ occurred only in the presence of AcNeu or pyruvate. These results suggest the participation of Schiff-base formation in the AcNeu lyase reaction.

DISCUSSION

Rutter [28] has classified aldolases into two classes, I and II, depending on the formation of a Schiff-base intermediate or the requirement for a metal ion cofactor. From the results obtained here (Table 2 and the other inhibition test), *E. coli* AcNeu lyase (AcNeu aldolase) should be classified as a Class I aldolase.

Among Class I aldolases, fructose 1,6-bisphosphate aldolases (EC 4.1.2.13), which possess a tetrameric structure, from a wide variety of animals and plants, have been studied in detail [26]. Their catalytic characteristics are as follows. (*a*) Participation of histidine residue(s) in the enzyme reaction has been observed in photo-oxidation experiments. The rate of photo-oxidation of the essential histidine residues is relatively independent of pH over the range 5.5–8.5, suggesting that these histidine residues are not readily protonated and probably exist in a hydrophobic environment [26]. (*b*) With respect to cysteine residues, fructose 1,6-bisphosphate aldolase is inactivated by low concentrations of Cu^{2+}, Ag^{2+} or *p*-chloromercuribenzoate [26], but it is not inactivated by treatment with alkylating reagents of thiol groups, such as iodoacetate and iodoacetamide [26]. These catalytic properties resemble those of *E. coli* AcNeu lyase described here (Fig. 5 and other inhibition test). These results suggest a similarity between the reaction mechanisms of these two kinds of aldolase, in spite of the difference in their subunit structures.

AcNeu lyase from *Cl. perfringens* has been purified and well characterized [10,11,29]. The *E. coli* enzyme studied here shows several similarities to the *Cl. perfringens* enzyme, but also some differences. AcNeu lyase from either source is a Class I aldolase and has similar properties with respect to pH optima, K_m values and substrate specificities. The native enzyme from *Cl. perfringens* exhibits a molecular mass of between 92000 [29] and 99200 Da [11]. The enzyme dissociates into subunits of molecular mass 50000 kDa, suggesting that it is composed of two identical subunits. On the other hand, the native enzyme from *E. coli* is shown to have a molecular mass of about 110000 Da. The enzyme dissociates into subunits of molecular mass 35000 Da, suggesting that the enzyme is composed of three identical subunits. These results indicate that the *E. coli* enzyme and the *Cl. perfringens* enzyme differ from each other in molecular size and quaternary structure. Although trimeric proteins are very rare, two other examples of trimers have been reported for related enzymes. For phospho-2-dehydro-3-deoxygluconate aldolase (EC 4.1.2.14) of *Pseudomonas putida*, the native molecular mass is 72000 Da and its subunit molecular mass is 24000 Da [30,31]. For 4-hydroxy-2-oxoglutarate aldolase

(EC 4.1.3.16) of *E. coli*, the native molecular mass is 64700 Da and its subunit molecular mass is 21000 Da [32]. Although their enzymic properties have not been characterized sufficiently, *E. coli* AcNeu lyase appears to resemble them with regard to their reaction mechanisms.

It appears likely that the primary physiological function of AcNeu lyase is to break down sialic acid into common metabolic intermediates, because it showed a high degree of specificity for sialic acid and was induced by it but not by pyruvate or *N*-acetyl-D-mannosamine, the primary degradation products of sialic acid [7]. *E. coli* AcNeu lyase activity was also regulated by product inhibition, as described here.

E. coli AcNeu lyase has some unique characteristics from the standpoint of enzymology, e.g. its trimer subunit composition and high heat-stability. More detailed studies, including analysis of its three-dimensional structure, will be necessary to clarify these points.

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