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The sialate pyruvate-lyase from pig kidney: Purification, properties and genetic relationship+

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For further insight into the structural relationship between mammalian and microbial sialate pyruvate-lyases, the enzyme from pig kidney was purified to homogeneity from the tissue homogenate by a heat precipitation step followed by anion exchange and Hydrophobic Interaction Chromatography or native gel electrophoresis, respectively. The pure enzyme preparation exhibited an about 1000-fold increase of specific activity compared to the supernatant after the first centrifugation and revealed a single band at 34–37 kDa after SDS-PAGE, which represents the monomeric form of the protein. While the native enzyme seems to be a trimer according to the molecular weight obtained by gel filtration (108 kDa), crosslinking with dimethylpimelimidate suggests it to be a tetramer. The lyase is optimally active at about 75 °C and in the pH range of 7.6 to 8.0 and belongs to the class I-aldolases [1], due to its non-requirement of metal ions and the presence of lysine as the main functional residue in its catalytic centre. These data are similar to those obtained with bacterial lyases. However, peptide fragments of this enzyme show less similarity to primary lyase structures of microbia than to those derived from expressed sequence tags of mammals.

Keywords: **sialate pyruvate-lyase, *N*-acetylneuraminate lyase, aldolase, pig kidney, purification, properties, mammalian lyases, bacterial lyases, primary structures, alignment**

Abbreviations: **DMAB, *p*-dimethylaminobenzaldehyde; DMB, diamino-4,5-methylenedioxybenzene; DMP, dimethylpimelimidate-dihydrochloride; DTNB, 5,5′-α-Dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol; EST, expressed sequence tag, cDNA sequences obtained after reverse transcription of mRNA; HIC, Hydrophobic Interaction Chromatography; IEP, isoelectric point; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid; Neu5,9Ac$_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu2en5Ac, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; OD, optical density; PA, polyacrylamide; DMAB, *p*-dimethylaminobenzaldehyde; PMSF, phenylmethylsulphonyl fluoride.**

Introduction

Sialic acids, the derivatives of neuraminic acid, represent a family of 44 naturally occurring monosaccharides generally bound in terminal position of the oligosaccharide chains of glycolipids or glycoproteins on cell surfaces, with widespread occurrence and functions, mainly in cellular and molecular recognition, adhesion, masking, and defense [2–4]. Some sialic acids are targets for pathogens, like the most common sialic acid *N*-acetylneuraminic acid for many bacteria, viruses and protozoa [4], *N*-acetyl-9-*O*-acetylneuraminic acid for e.g. influenza C virus [5], or they are indicators of diseases, like *N*-glycolylneuraminic acid for some human tumors [2,3].

Sialate pyruvate-lyases (*N*-acetylneuraminate lyases, EC 4.1.3.3) are the terminal enzymes in sialic acid metabolism [6]. They cleave sialic acids into the corresponding *N*-acylmannosamines and pyruvate; the main substrate is Neu5Ac. With regard to the functions of these catabolic enzymes, in procaryotes they mainly provide pyruvate as carbon and energy source and regulate the intracellular sialic acid level [7], while they may prevent recycling of sialic acids in mammalian species [6]. In the laboratory, sialate pyruvate-lyases are used for the synthesis of natural and non-natural sialic acids using mannosamine derivatives [8–10] and in sialic acid analysis they are applied for the specific identification of distinct peaks as sialic acids especially on fluorescent HPLC chromatography [11] by a previous incubation with the enzyme [12].

Lyase activity is found in a variety of microbial species [13]. The enzymes from *Escherichia coli* [14–17] and *Clostridium perfringens* [18–21] have been purified, characterized and the corresponding genes cloned. Also the X-ray

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structure of the *E. coli* enzyme has been elucidated [22]. The nucleotide sequences of the lyases from *Haemophilus influenzae* [23], *Trichomonas vaginalis* [24], and a partial sequence of *Clostridium tertium* lyase [25] are also known. Compared to the close relationship among the lyase sequences of *H. influenzae, T. vaginalis* and *C. perfringens,* the enzyme from *E. coli* seems to be only weakly related to each of these three sequences. Thus two groups of microbial sialate pyruvate-lyases can be distinguished [21].

Apart from microbial species, these lyases seem to exist generally in mammals, mainly in kidney and liver [6,26]. Attempts to purify the porcine [26,27] and bovine [28] enzymes were made. So far, there are not enough structural data available for a general comparison between these two and the microbial lyases, which stimulated us to start a new approach on the sialate pyruvate-lyase from pig kidney. A part of the results obtained has been published as a symposium abstract [29].

Materials and methods

Substances

All chemicals for which no special source is given were purchased from Merck, Germany. Sialic acids had been prepared according to [12] and [30].

Determination of enzyme activity, protein concentration and enzyme purity

Enzyme activity was determined by three different test systems: the Morgan-Elson assay for the detection of *N*-acylhexosamines [31]; a test system for the determination of pyruvate (LDH/NADH-assay) [18]; or fluorimetric HPLC according to Hara et al. [11] for determination of the decrease of the sialic acid amount during the lyase reaction. Controls and calibration curves were made using sodium pyruvate, *N*-acetylmannosamine, or Neu5Ac (Snow Brand Milk Products, Japan) solutions. In most cases, the first test was used, due to the relatively high number of samples that can be measured simultaneously. The following slightly modified standard procedure was applied: 10 µl of enzyme preparation, 2.5 µl of 0.1 M Neu5Ac and 37.5 µl of 250 mM potassium phosphate buffer, pH 7.4, were incubated at 37 °C for 1 h. The reaction was stopped by heating at 96 °C for 4 min and the samples were put on ice. 20 µl of 0.8 M potassium borate buffer, pH 9.1, and 50 µl of water were added and the whole batch was heated at 96 °C for 12 min and cooled on ice, before 1 ml of a freshly prepared solution of 1 volume DMAB stock solution (10 g DMAB in 87.5 ml acetic acid and 12.5 ml 37% HCl) in 9 volumes glacial acetic acid was added. After a further incubation for 15 min at 37 °C, the amount of acylhexosamines was determined photometrically at 585 nm. The LDH/NADH assay was carried out after incubation at 37 °C and the first heating step at 96 °C was performed as described for the previous assay. Then 750 µl water, 125 µl 250 mM potassium phosphate buffer, pH 7.4, and 75 µl of a 2 mM NADH (Boehringer Mannheim, Germany) stock solution in this buffer were added and the absorbance of the whole solution was measured photometrically at 340 nm. After the addition of 1 µl LDH suspension (Boehringer Mannheim) (3.7 U), the amount of pyruvate formed was determined from the OD_{340nm} difference. For kinetic measurements, the decrease of OD_{340nm} was determined during incubation of the whole enzyme batch including NADH and LDH in the cuvette at 37 °C. Fluorimetric HPLC was carried out as described [11] using 1 µg of Neu5Ac or a sialic acid mixture for calibration (see below).

Total protein was determined by the Bradford assay [32] using the BioRad (Germany) test kit. As a control of enzyme purity, SDS-PAGE was performed [33], followed by staining with Coomassie Brilliant Blue R 250 (Serva, Germany) [34] or silver [35]. When using Coomassie Blue, the PA-gels were normally destained in 10% acetic acid / 40% ethanol. In the case of the protein band cut out for peptide sequencing (see below), only 10% acetic acid was used instead.

Purification of the enzyme

Pig kidney (400 g) from the local slaughter house was sliced into small pieces and freed from connective tissue. Two parts (w/w) of 25 mM potassium phosphate buffer, pH 7.0, containing 1 mM PMSF (Sigma, USA), were added. The material was homogenized and centrifuged for 1 h at 2 °C and 12000 × **g.** Sodium pyruvate was added to the supernatant to a final concentration of 40 mM. After heat precipitation for 12 min at 80 °C in a microwave apparatus and following centrifugation for 10 min at 2 °C and 40000 × **g,** the supernatant containing up to 1 g of protein was mixed with 0.5 volume water and applied to a Q-Sepharose column (30 ml, h = 3 cm, 150 ml/h) equilibrated with 5 column volumes of 33 mM potassium phosphate buffer, pH 7. Proteins were eluted stepwise at 33 mM (10 column volumes) and 67 mM (15 column volumes) potassium phosphate buffer, pH 7. The 67 mM-fraction, containing the major part of enzyme activity, was carefully mixed with ammonium sulfate and K_2HPO_4 to a final concentration of 1.8 M and 40 mM, respectively, and applied to an ethylagarose column (10 ml, h = 3 cm, max. 75 ml/h). After stepwise elution with 1.4 M (10 column volumes) and 1.2 M (10 column volumes) of ammonium sulfate (from a stock solution of 3 M ammonium sulfate and 50 mM K_2HPO_4, pH 7.0), two different ways were taken for further purification of the 1.2 M fraction:

A second HIC run on a ET20 column (perfusion chromatography on a BioCAD-Workstation, PerSeptive Biosystems, Inc., Germany) with elution steps using 1.4 M and 1.25 M ammonium sulfate, allowed quick purification. After the enzyme solution had been concentrated to 200 µl (200 µg protein) by pressure dialysis, it was subjected to a

SDS-PAGE and the protein band cut out after Coomassie Blue staining (see above). Peptide fragments of the enzyme were produced and sequenced by the Wittmann Institute of Technology (Germany). The fragments were obtained by digestion of the enzyme protein with endoproteinase C (Boehringer Mannheim), separated by narrowbore-HPLC using the SMART-System (Pharmacia) and five of the resulting peptides were sequenced by a Knauer Protein Sequencer 910.

Alternatively, the ethylagarose step was followed by native PAGE (same conditions like SDS-PAGE, but without SDS and 4% stacking and 8% resolving gel). After cutting the gel into about 20 slices, the enzyme was eluted in 250 mM potassium phosphate buffer, pH 7.6, containing 1 mM DTT. Ammonium sulfate (0.5 volume, 3 M) solution was added to the active fractions for storage at −20 °C.

Determination of enzyme properties

If not described otherwise, investigations were carried out with the ET20-purified enzyme. Determination of the mass of the native lyase was carried out by gel filtration on a Sephacryl S-200 (50 µg of the purified sample applied; d = 1.5 cm, h = 80 cm, 10 ml/h) or a Superose 6 HR column (10 µg of purified sample applied; d = 1 cm, h = 30 cm, 0.4 ml/min) (both from Pharmacia) in 150 mM KCl with 50 mM potassium phosphate buffer, pH 7. The columns were calibrated using the Sigma MW-GF-200 protein standard kit. Crosslinking was carried out by incubating the enzyme (5 µg) in a solution of 1 µg/µl DMP at room temperature for 1 h and detection of the resulting protein bands by SDS-PAGE.

The temperature optimum was estimated using the Morgan-Elson assay, by incubation for times between 10 and 30 min at 30° up to 105 °C for the pig kidney lyase, and an incubation time of 10 min for the recombinant lyase from *C. perfringens*. Determination of the pH optimum was carried out by using the same assay. Incubation was performed for 30 min at 37 °C or 75 °C in 0.25 M potassium phosphate buffers, ranging from pH 5.5 to 8.5 (measured at room temperature).

The IEP was measured by focussing the enzyme purified by native PAGE as the last step in a 16 ml Rotofor cell (BioRad) as described by the manufacturers.

Kinetic data were determined at 37 °C and 75 °C with Neu5Ac concentrations of 0.5, 1, 2, 4, 6, 8, 10 and 20 mM and incubation times of 10–15 min (Hanes-plot [36]), by the Morgan Elson assay or only at 37 °C, with the LDH/NADH assay. The latter assay was also used for up to one hour at 37 °C in 250 mM potassium phosphate buffer, pH 7.4 to determine the substrate specificity with 5 mM Neu5Gc, Neu5,9Ac_2 (both isolated from porcine and bovine submandibular gland mucin, respectively), 7-deoxy-Neu5Ac (a kind gift of the late Professor Erich Zbiral, Vienna), Neu2en5Ac (Boehringer Mannheim) and a mixture of α2,3- and α2,6-sialyllactose (from cow colostrum) instead of Neu5Ac. The reactivity with Neu4,5Ac_2 was determined in a sialic acid mixture with Neu5Ac and Neu5Gc (isolated from guinea pig serum; the ratio of Neu5Ac:Neu5Gc:Neu4,5Ac_2 was roughly 1:6:3) by fluorimetric HPLC.

The influence of several metal ions was measured by adding 1 mM of divalent or 10 mM of monovalent cations according to [20] to the incubation mixture. Generally, the Morgan Elson assay was used here, but in the presence of Cu^{2+} the LDH/NADH assay had to be used and in the case of Hg^{2+} fluorimetric HPLC was applied, due to the disturbing effect of Cu^{2+} on the Morgan Elson assay and of Hg^{2+} on both photometric tests.

Determination of the influence of amino acid-modifying reagents on enzyme activity in most cases was carried out by preincubation of the enzyme for 15 min with the corresponding reagent at 37 °C, followed by incubation with the substrate Neu5Ac. Succinanhydride and *N*-bromosuccinimide (Sigma) were used as 1 M stock solutions in acetone, and *p*-chloromercuribenzoate as 0.1 M stock solution in 0.1 M NaOH. Preincubation with *N*-acetylimidazole (Sigma) was carried out for 1 h at room temperature in 50 mM potassium phosphate buffer, pH 7.5. Preincubations of the enzyme with $NaBH_4$ (1 mg/ 50 µl) according to Aisaka et al. [16] were carried out for 1 h in 250 mM potassium phosphate buffer, pH 6, in the presence or absence of 0.1 mM sodium pyruvate or Neu5Ac. Remaining activity was determined after dialysis against 250 mM potassium phosphate buffer, pH 7.5.

Alignment

Microbial lyase sequences were taken from EMBL or Swiss Prot databases: *E. coli* P06995 [15], *H. influenzae* L44787 [23], *T. vaginalis* U 35878 [24] and *C. perfringens* Y12876 [21]. EST sequences from mouse (WW162738 [37]) and human fetal heart (A709930 [38]) were derived from EMBL databases and translated into protein sequences. The alignment was checked by the program husar 8.1 (Germany).

Results

Purification

According to the procedure described, the sialate pyruvate-lyase from pig kidney was enriched about 1000-fold in five steps (Table 1) and showed one single band after SDS-PAGE (Fig. 1A) and Coomassie Blue or silver staining. The maximum specific activity was about 1.5 U/mg protein under the standard assay conditions. The lyase protein represented the main band after HIC on ethylagarose. After a second HIC on an ET20 column (perfusion chromatography), the obtained protein (Fig. 1B) was concentrated and cut out of the gel after SDS-PAGE for peptide sequencing.

Table 1. Purification of the sialate pyruvate-lyase from pig kidney

Step	*Volume [ml]*	*Protein conc. [mg/ml]*	*Enrichment*	*Yield [%]*	*Spec. act. [mU/mg prot.]*
Supernat. after homogenisation	2300	20	1	100	1.5
Heating	2000	0.8	11	40	17
Q-Sepharose chromatography	680	0.25	27	10	41
Ethylagarose chromatography	129	0.05	286	4	429
Perfusion chromatogr. (ET20) or	0.58	0.17	922	0.2	1383
Native gel electrophoresis	9	0.1	1032	2	1548

The following sequences of five peptides were obtained: IGADGI, DNLINF, IRAEEL, IPTFQG and TNQMLE.

Properties

The enzyme properties are summarized in Table 2. The molecular mass of the purified lyase monomer after SDS-PAGE is 34 kDa (Sigma-standard) or 37 kDa (Gibco-standard) (Figures 1 and 2A). The molecular mass of the native enzyme according to gel filtration experiments on Sephacryl S-200 and on Superose-6 (Fig. 2B) is about 106 and 110 kDa, respectively. Thus, the enzyme appears to be a trimer. On the other hand, crosslinking with DMP resulted in a band of about 140 kDa after SDS-PAGE, which suggests the sialate pyruvate-lyase to be a tetramer (Fig. 2A). The cleaving reaction works optimally at pH values between 7.6 and 8.0 (Fig. 3A) and a temperature of about 75 °C (Fig. 3B). In the presence of ammonium sulfate (240 mM), the enzyme is about twice as active at the respective temperature as in the absence of this salt (Fig. 3B). The Michaelis-Menten constant for Neu5Ac was determined to be 1.7 mM at 37 °C and 1.0 mM at 75 °C with a V_{max} of 2.3 and 7.1 U/mg protein, respectively, according to a Hanes Plot [36]. Concentrations of 1 mM of Cu^{2+}, Fe^{2+}, or Hg^{2+}

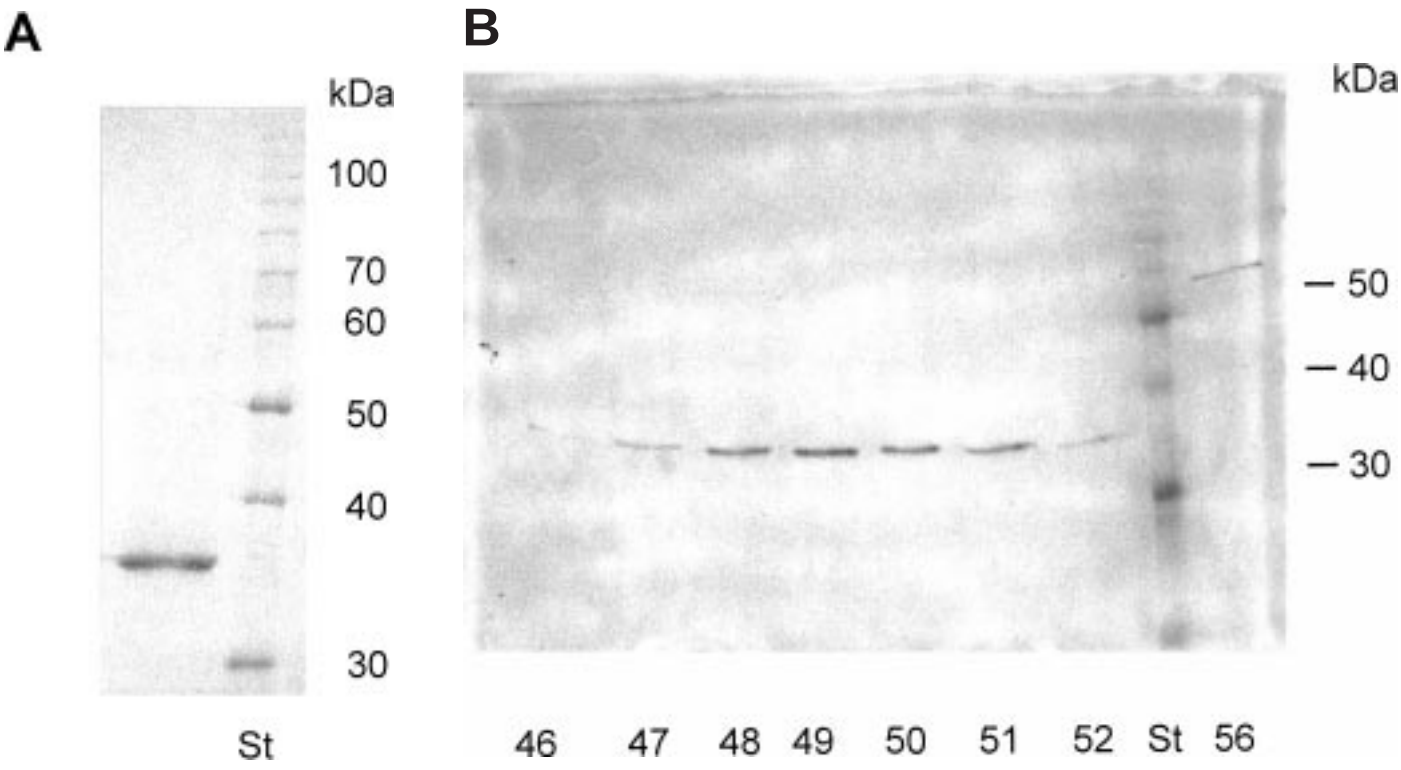

Figure 1. Purity and molecular mass determination of pig kidney sialate pyruvate-lyase. (A) SDS-PAGE (Coomassie Blue-stained) of the enzyme finally purified by native gel electrophoresis and compared with the Gibco protein standard (St). (B) SDS-PAGE (silver-stained) of fractions from the final HIC step on POROS ET20, containing the maximum enzyme activity (fractions no 45–52) and a following non-active protein peak (fraction no 56), compared with the Gibco standard (St).

Table 2. Properties of the sialate pyruvate-lyases from mammalian and bacterial sources

Property	*Pig kidney*	*Escherichia coli*	*Clostridium perfringens*
M_r [kDa] (denat.)	34–37[a]	33–35[a][14,16]	32.3[b][21]
M_r [kDa] (native)	110[c]/140[d]	98–105[c][14,16]/132[e]	99.2[f][20]
pH-optimum	7.6–8.0	7.7[14]; 6.5–7 [16]	7.2[20]
Temp. Optimum [°C]	75	75 [14]; 80 [16]	65–70
Isoelectric point	5.5	4.5 ± 0.1 [16]	4.7 [20]
Thermostability	Yes	Yes [14,16]	Yes [39]
Metal ions required	No	No [14]	No [20]
K_M at 37 °C [mM]	1.7	3.6 [14]; 3.3 [16]	2.8 [20]
V_{max} at 37 °C [U/mg]	2.3	71.4 [16]; 154.5 [14]	n.d.
Substrate specificity (relative cleavage rate [%])			
N-acetylneuraminic acid	100	100 [16]	100 [40]
Neu5Gc	47	20 [16]	93 [40], 65 [18]
Neu5,9Ac_2	45		
Neu4,5Ac_2	0–10		
Neu2en5Ac	0		0 [40]
7-desoxy-Neu5Ac	5		0 [43]

[a]SDS-PAGE; [b]calculated from gene sequence; [c]gel filtration; [d]crosslinking with DMP; [e]calculated from monomeric size [14] and X-ray data [22]; [f]different methods, e.g. gel filtration; n.d., not determined

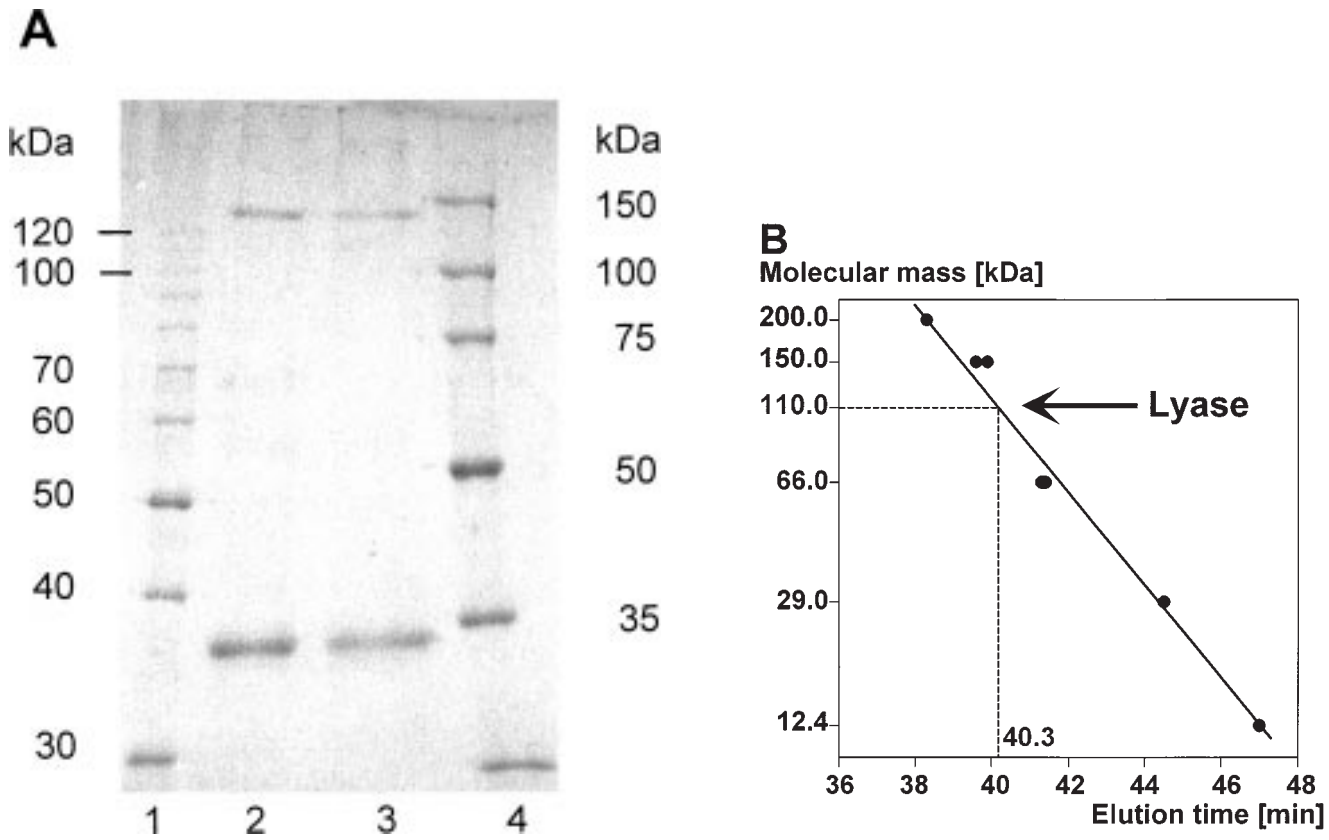

Figure 2. Determination of the molecular mass of the native pig kidney sialate pyruvate-lyase and the number of subunits. (A) Estimation of the mass and number of subunits by crosslinking with dimethylpimelimidate followed by SDS-PAGE (Coomassie Blue-stained): 1, Gibco protein standard; 2, cross-linking experiment: lyase monomer, not crosslinked (lower band, 34–37 kDa), and crosslinked enzyme (upper band, about 140 kDa); 3, like 2, electrophoresis without dithiothreitol; 4, Sigma protein standard. (B) Determination by gel filtration of the native enzyme mass on Superose-6 with various standard proteins.

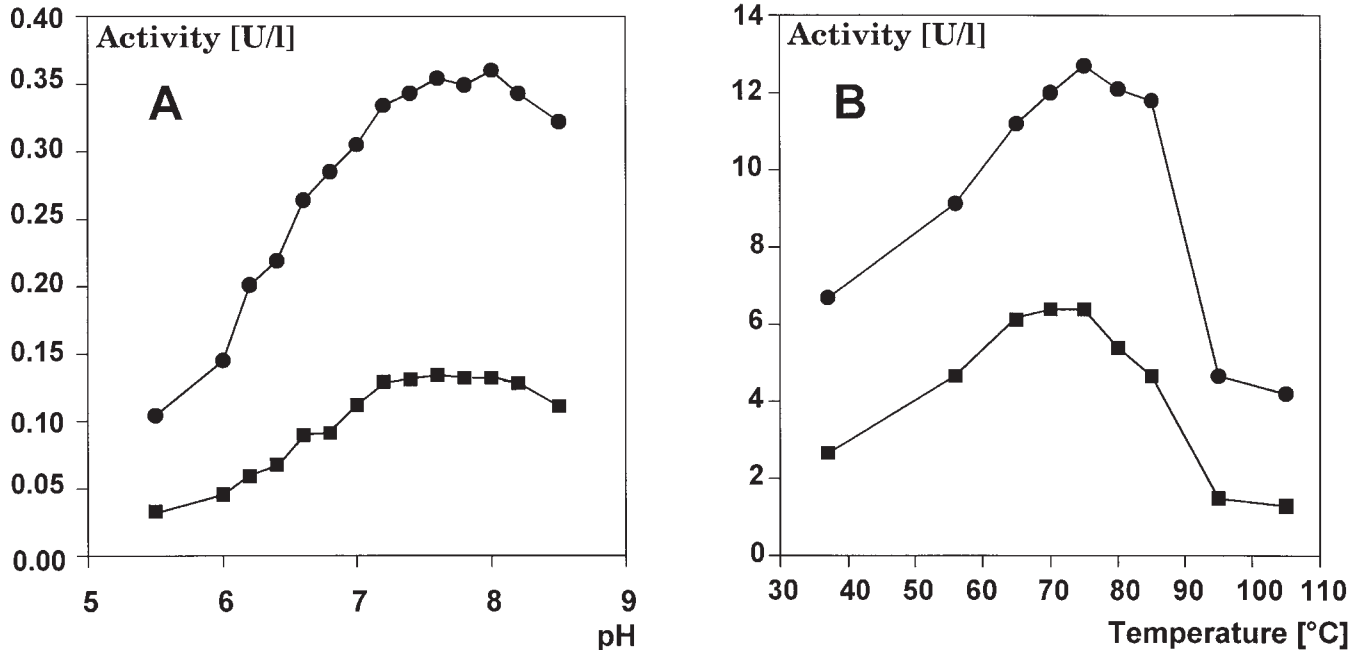

Figure 3. Optimum pH (A) at 37 °C (●) and 75 °C (■) of the sialate pyruvate-lyase after the second HIC step. Temperature optimum (B) with (●) and without (■) ammonium sulfate (240 mM); determined with the same enzyme sample.

decreased the enzyme activity by 20, 40 and 45 %, respectively.

Amino acid-modifying reagents

Several substances were added to the enzyme solution before the catalytic reaction was started, in order to investigate the influence of special amino acid residues on the catalytic mechanism. The data are given in Table 3. The values are means of each five measurements. Less than half of the enzyme activity is left after addition of the SH-modifying reagent *p*-chloromercuribenzoate (1 mM). A distinct inhibitory effect is also exerted by *N*-bromosuccinimide and *N*-acetylimidazole, which act on histidine, tyrosine and tryptophane. The strongest inhibition, however, was achieved with the four lysine-modifying chemicals summarized in Table 3, especially by the combination of substrate and sodium borohydride.

Alignment

In a first attempt, the peptide fragments sequenced from the pig kidney lyase could not reliably be aligned to the known lyase sequences of *E. coli, C. perfringens, T. vaginalis* and *H. influenzae* (for their total alignment see [21]). This was, however, possible after implementation of ESTs from mouse (AA162738) and human foetus (W79930) ([37,38]; Dr. P. Colman and Dr. M. Lawrence, pers. comm.), which show similarities to the microbial sialate pyruvate-lyases.

The partial mammalian sequences have about 40% of the length of the microbial lyases. From these ESTs, the amino acid sequences were deduced and aligned to the microbial and the porcine peptide sequences (Fig. 4). Three of the five porcine peptides obtained are completely identical to the mammalian sequences. However, one peptide sequence differs in one position to one and in another position to both of the corresponding areas in the EST-sequences. The first three amino acids of the fifth peptide fit to corresponding residues at the end of the sequence from foetal heart.

Discussion

Purification and properties

Among the purification steps leading to an about 1000-fold increase of the specific enzyme activity, heating and ethylagarose chromatography were most efficient. The presence of sodium pyruvate had a protecting effect on the catalytic centre during the heating step [39]. The chromatography with Q-Sepharose removed among other proteins albumin and most non-protein substances. While the second HIC step was fast, the alternatively used native gel electrophoresis gave a 10-fold higher yield of the pure enzyme protein (Fig. 1A and Table 1).

Most properties determined for the porcine kidney lyase are in good agreement with the results for the two microbial lyases studied (Table 2). Thus, the optimum pH of the lyase lies in the neutral range in all three species, which

Table 3. Influence of amino acid-modifying reagents on the activity of the sialate pyruvate-lyase from pig kidney

Reagent	*Conc. [mM]*	*Remaining activity [%]*	*Modified group*
Control	0	100	
p-Chloromercuribenzoate	0.1	65	SH
	1	40	SH
DTNB	1	65	SH
	10	23	SH
Dithiothreitol	1	121	S-S
	10	64	S-S
$NaBH_4$ + pyruvate (0.1 mM)	263	8	Lys
$NaBH_4$ + Neu5Ac (0.1 mM)	263	8	Lys
KCN	10	39	Lys
Succinanhydride	1	42	Lys
N-Bromosuccinimide	0.1	50	His, Tyr, Trp
N-Acetylimidazole	100	10	Tyr
PMSF	100	80	Ser

could be a hint for a cytosolic location of the enzyme. A special feature, which was also found for both the microbial and the mammalian lyases, is the high temperature optimum around 70 °C, and the thermostability, especially in the presence of pyruvate. Only little is known about the structural basis of this property. Interestingly, thermostability has often been found for tetrameric proteins. Perhaps, salt bridges between e.g. glutamate residues and Ca^{2+} contribute to it. The stabilizing effect of ammonium sulfate on enzyme activity (Fig. 3B) could be due to an increase in the hydrophobicity of the environment, which results in small conformational changes and a higher stability of the enzyme molecule. The kinetic data at 75 °C have so far not been determined for any sialate pyruvate-lyase. The difference in V_{max} at 37 °C and 75 °C results from the difference in the incubation temperature; the lower K_M value at 75 °C may not be interpreted as a result of higher affinity to the substrate, since the temperature dependence of each kinetic parameter influencing the idealized Michaelis-Menten kinetics has to be considered.

The molecular mass of the native porcine enzyme of on average 108 kDa was only obtained by using the purified enzyme, because otherwise the enzyme seemed to interact with other proteins, thus preventing reliable results. Both gel filtration and crosslinking experiments leading to the assumption of a trimeric or tetrameric structure are in good agreement with gel filtration and X-ray analysis data of the enzyme from *E. coli* [14,16,22]. The values obtained by gel filtration may be misleading because of the special form of the tetrameric protein [22]. On the other hand, there is a significant difference to earlier results for the bovine and the porcine lyase [27,28] in mass and subunits. These mammalian lyases had been characterized as monomers of 55 and 58 kDa, respectively. A similar value of 50 kDa, however, per subunit was earlier reported for the enzyme from *C. perfringens* [20] having a dimeric structure. During the work presented here, no hint on a porcine sialate pyruvate-lyase with this molecular mass was found. Not totally purified enzyme, use of a too low ionic strength during gel filtration, or misinterpretation of standards may be possible reasons for these earlier, deviating findings.

Concerning the data listed in Table 2, there was no great difference between these and those presented in [26] and [27] and it is possible to confirm the conclusion made in [27] of a high similarity between the properties of the porcine and the bacterial lyases: Although the substrate specificity has different percentual values, a similar tendency was mainly determined for the enzyme of *C. perfringens* [40,41]; it shows that free hydroxyls at positions 2, 4 and 7 are important for catalysis by the enzyme. The significance of the latter position can be seen from the low splitting rate of 7-desoxy-Neu5Ac.

The enzyme activity did not depend on metal ions, thus confirming that the kidney lyase is not a metal-dependent class II-aldolase according to the classification of Rutter [1].

Amino acid-modifying reagents

The inhibitory effect of *p*-chloromercuribenzoate, DTNB (Table 3) and of heavy metal ions on enzyme activity are hints on the involvement of a SH-group in the catalytic mechanism. The inhibition by reduction of the enzyme-substrate complex with pyruvate or Neu5Ac by $NaBH_4$ much suggests lysine as a catalytic residue, forming a Schiff base with the substrate. Some of the experiments with amino

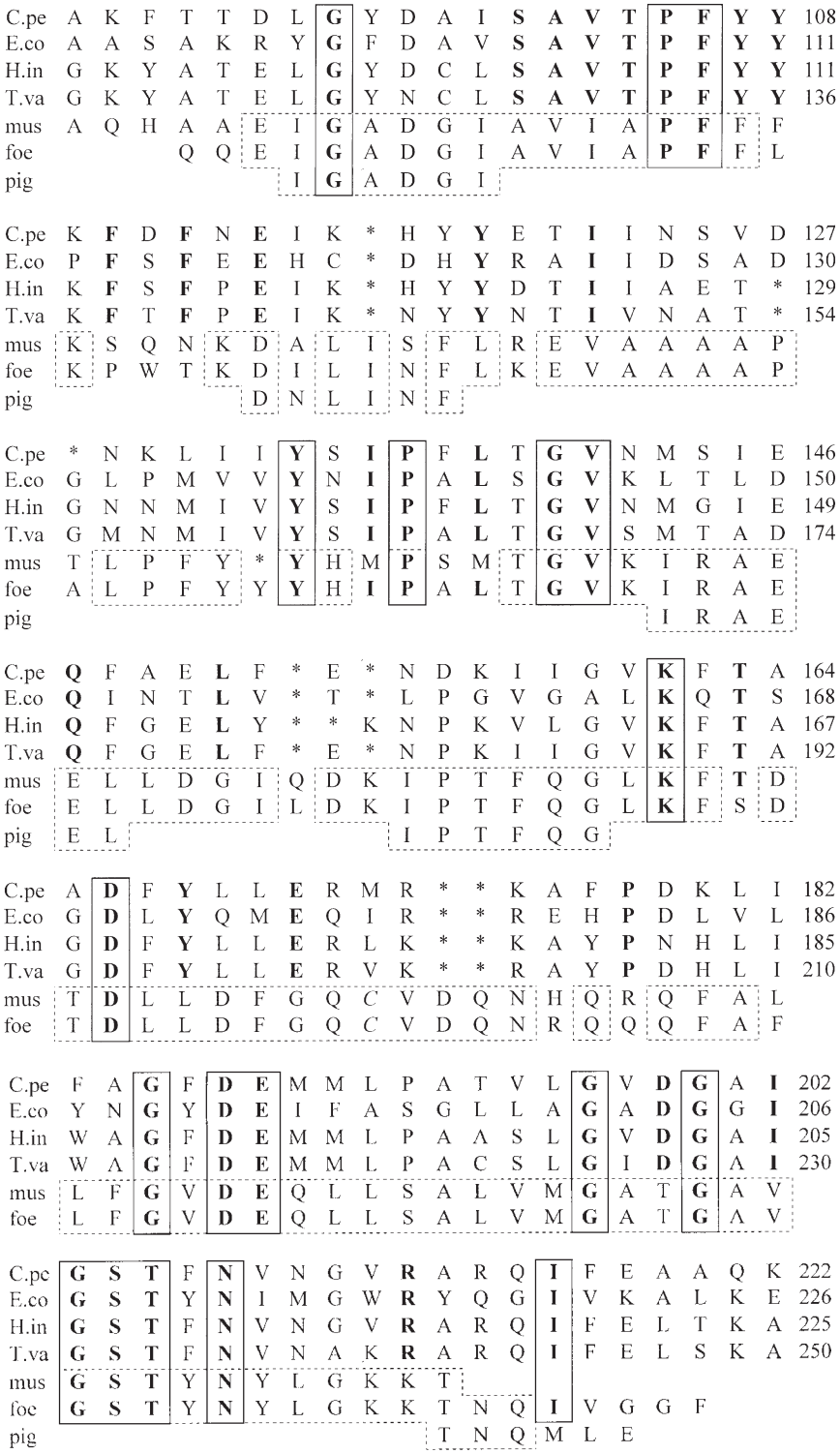

Figure 4. Alignment of the amino acid sequences from mammalian and microbial sialate pyruvate-lyases. C.pe: *Clostridium perfringens;* E.co: *Escherichia coli;* H.in*: Haemophilus influenzae;* T.va: *Trichomonas vaginalis;*. mus: mouse (deduced from EST; accession no. AA162738); foe: human foetal heart (deduced from EST; accession no. W79930); pig: sequenced peptides from pig kidney lyase; *no corresponding amino acid in this sequence;

X identical amino acids in all bacteria

X identical in all mammals

X identical in all species

C cysteine residue conserved in mammalian sequences

acid-modifying reagents had also been performed earlier with the porcine lyase [27] and gave similar results. Furthermore, a hint on the involvement of histidine mainly by using Rose Bengal and diethylpyrocarbonate was provided [27]. These data correspond well with those obtained for the enzyme from *C. perfringens* [19]: A SH-group is proposed to be involved in the mechanism, and a lysine residue to form a Schiff base (class I-aldolase) with the pyruvate part of the substrate molecule, which can be modified by KCN or $NaBH_4$. A histidine and/or a tyrosine residue might be important for the catalytic mechanism as well (see below).

Together with the data obtained for substrate specificity, these observations made it possible to design a model for the catalytic mechanism [2,20; and Fig. 5]. It was originally developed for the enzyme from *C. perfringens:* According to this hypothesis, the reaction starts with a nucleophilic attack of a histidine residue on the OH-proton at C-4 of the sialic acid molecule, which exists as a closed pyranose ring. After cleavage of the C-C-bond between C-3 and C-4, a lysine residue forms a Schiff base with C-2 and induces opening of the semiacetal bond of the Neu5Ac ring, which results in the release of the hexosamine part of the molecule. Alternatively, out of the equilibrium between the closed ring and open chain form of the substrate, the latter might react with the lysine residue of the enzyme first to form the Schiff base, and then the nucleophilic attack on the proton of the hydroxyl at C-4 takes place [42]. Furthermore, an amino acid can be postulated to be present in the active site, which would function as an acid in the removal of a water molecule from C-2 [2] (Fig. 5). The similarity in the behaviour against modifying reagents and in substrate specificity allows the prediction of a corresponding reaction mechanism for the porcine lyase.

Alignment

From the alignment of five lyases and two ESTs, three conclusions can be deduced: First, comparison of the two EST-derived putative partial lyase sequences with the authentic porcine peptides strongly confirms the assumption that these gene fragments really are parts of sialate pyruvate-lyases. From the high degree of similarity between these sequences it can be deduced that mammalian sialate pyruvate-lyases seem to be a closely related group of enzymes that can be well distinguished from the microbial species. According to the X-ray structure of the *E. coli* enzyme [22], a lysine residue is found in the catalytic centre. This lysine confirms the experiments with chemical modulation of enzyme activity described here. However, no histidine was found, but tyrosine exists in a corresponding position. This fits to the data from the alignment, where Lys-165 and Tyr-137 (position in *E. coli*) are conserved in all lyases as well as in the mammalian EST-sequences. This means that the proposed catalytic mechanism discussed has to be modified in a way that the nucleophilic attack on the hydroxyl proton at C-4 of the sialic acid molecule leading to the retro-aldol-reaction could be carried out by the phenolic group of the tyrosine residue, while the substrate at C-2 is fixed at lysine via a Schiff base, followed by ring opening (Fig. 5). However, according to a model proposed by Smith et al. [44], the abstraction of the proton from the C-4 hydroxyl occurs through the carboxylate part of the substrate, to which tyrosine is hydrogen-bonded. The lack of a cysteine residue in the active site of the *E. coli* enzyme crystal structure is in contrast to the experiments with amino acid-modifying reagents and the inhibition of enzyme activity by heavy metal ions. In addition, regarding the full length (or as far as already known) sequences of all available lyase primary structures, no cysteine was found to be conserved throughout all of them. Solely in the microbial lyase primary structures [21] a conserved cysteine is present in the C-terminal region beyond the end of the EST sequences. Another cysteine residue is conserved only in the EST-sequences. Perhaps, SH-groups are not directly involved in the catalytic mechanism, but have a more general function for the correct conformation of the protein. On the other hand, the addition of mercaptoethanol in SDS-PAGE had no effect on the migration behaviour of the porcine enzyme. Therefore, disulfide bonds at least do not play a role in the linking between the enzyme subunits.

Because of similarities in the chemical and enzymatic behaviour and in structural data, a closely related reaction mechanism may be predicted for both mammalian and microbial lyases. Such a mechanistic uniformity corresponds well to the recognition of a family of enzymes structurally and functionally related to sialate pyruvate-lyase, e.g. dihydrodipicolinate synthase [42]. Of course, all these conclusions have to be confirmed by crystallisation of the porcine lyase and by cloning of its gene, which are now in progress.

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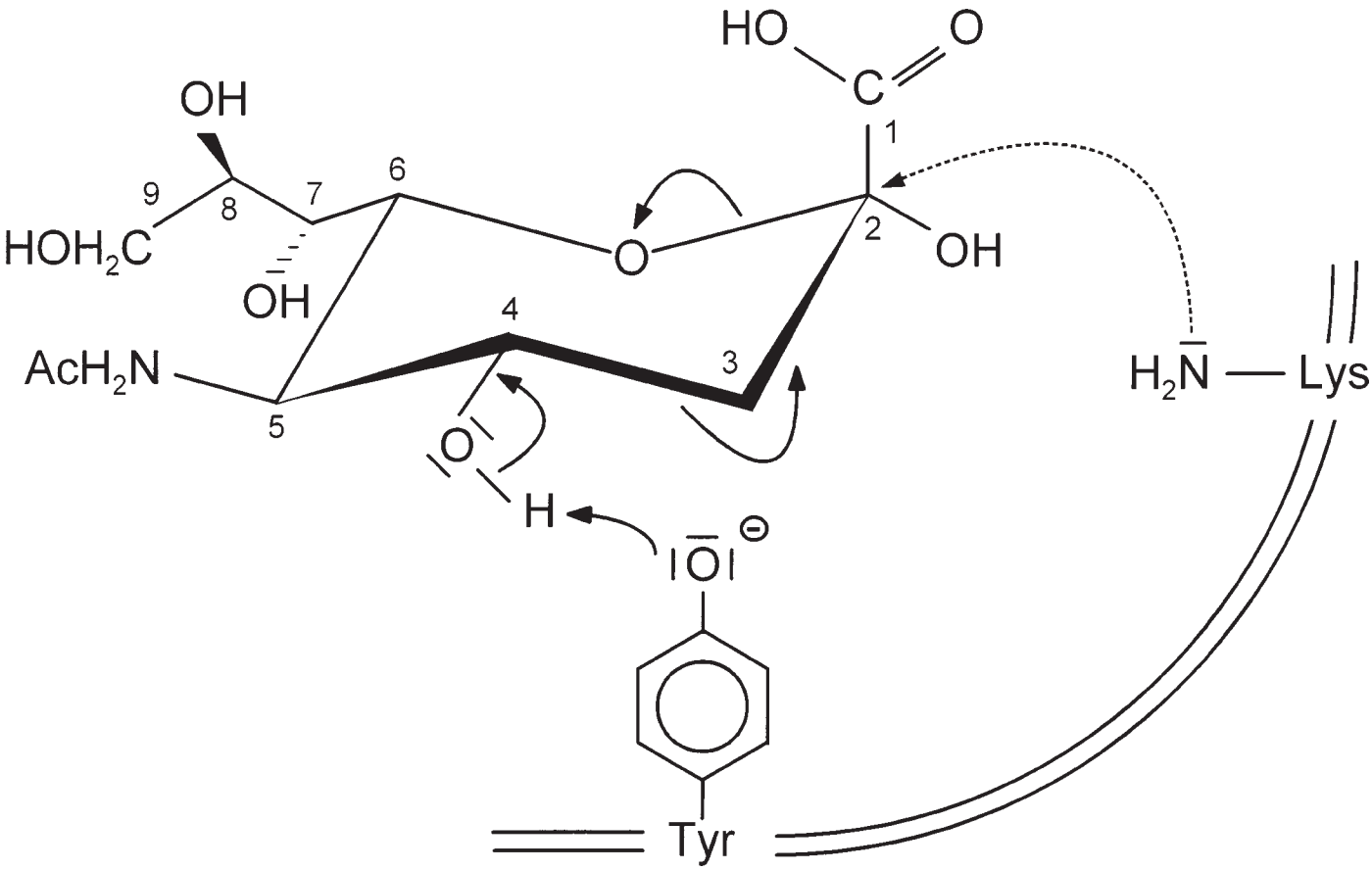

Figure 5. Mechanism of the catalysis of sialate pyruvate-lyases. This simplified reaction scheme is proposed on the findings described here and in an earlier model published in Ref. 2. After retro-aldolization is initiated by an attack of the tyrosine electron pair to the hydroxyl at C-4, the sialic acid is bound as a Schiff base to a lysine residue, followed by an opening of the hemiacetal bond of the pyranose ring and release of the acylmannosamine part. For further details see the text.

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