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The Reaction of *N*-Acetylneuraminate Lyase with Chloropyruvate

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Chloropyruvate, like bromopyruvate, rapidly inactivates *N*-acetylneuraminate lyase at pH 7.2. At 5°C, 0.5 mM-chloropyruvate reacted with the enzyme about ten times as fast as bromopyruvate. In contrast, at pH 6.0 and 9°C, chloropyruvate reacted with *N*-acetylcysteine seven times more slowly than bromopyruvate. A brief (2 min) incubation of the enzyme with 1.0 mM-chloro[^{14}C]pyruvate gave an inactive enzyme in which 4.5 cysteine residues were alkylated per molecule of enzyme. This corresponded to the number of [^{14}C]pyruvate residues (3.7) bound to the enzyme by borohydride reduction of the [^{14}C]-pyruvate complex, and confirmed the previous suggestion that there is one 'essential' cysteine residue per active site. It is suggested that, for this enzyme, chloropyruvate can be selectively used to alkylate the active-site residues, whereas bromopyruvate cannot. The apparent molecular weight of the enzyme prepared by two slightly different methods was approx. 100000 or 250000. The latter value has been used to calculate the number of pyruvate residues bound to the enzyme.

We have previously reported the inactivation of *N*-acetylneuraminate lyase (EC 4.1.3.3) from *Clostridium perfringens*, a typical Schiff-base aldolase, by bromopyruvate (Barnett *et al.*, 1971). The inactivation was accompanied by reaction with cysteine residues in the enzyme. The reaction was not specific for active-site residues, because approximately 20 cysteine groups were attacked per molecule of enzyme, whereas only four pyruvate molecules were bound to the active-site lysine by borohydride reduction of the pyruvate–enzyme Schiff-base intermediate complex.

Bromopyruvate has been widely used in active-site studies as an alkylating analogue of pyruvate. Some of the more successful applications have been to lactate dehydrogenase (Berghäuser *et al.*, 1971), malate dehydrogenase (decarboxylating) (Chang & Hsu, 1973) and 2-keto-6-phosphogluconate lyase (Meloche, 1970; Meloche *et al.*, 1972). Bromopyruvate is also a powerful non-specific alkylating agent and has been used as such (Rashed & Rabin, 1968; Baker & Rabin, 1969). The non-specific action competes with the active-site-directed properties for some enzymes, making the assignment of the reactive active-site residues more complicated. Examples of this behaviour were found with *N*-acetylneuraminate lyase (Barnett *et al.*, 1971), aspartate aminotransferase (Okamato & Morino, 1973) and the pyruvate dehydrogenase complex (Maldonado & Kyung-ja, 1972).

Because bromopyruvate has proved to be less specific as a pyruvate analogue than had been hoped, it seemed possible that the related chloropyruvate might prove more successful. This compound would be expected to react more slowly with peripheral thiol groups, and yet by virtue of its slightly smaller halogen atom might bind more strongly and react more readily with pyruvate-binding sites. We have therefore investigated the properties of chloropyruvate as an inactivator of *N*-acetylneuraminate lyase.

Materials and methods

N-Acetylneuraminate lyase (EC 4.1.3.3) (68 units/mg of protein) was prepared from *Cl. perfringens* (N.C.I.B. 8875, A.T.C.C. 10543) as described previously (Barnett *et al.*, 1971), except that a further purification step on DEAE-cellulose was added before the final chromatography on Sephadex G-200 (see DeVries & Binkley, 1972). DeVries & Binkley (1972) claim that their preparation with 88.5 units/mg of protein was pure.

Chloropyruvic acid was prepared by the action of SO_2Cl_2 on redistilled pyruvic acid as described by Cragoe & Robb (1960). The compound was distilled (b.p. 76°C/6 mmHg) and crystallized as a hygroscopic solid, m.p. 51–52°C [Garino & Muzio (1922) give m.p. 45°C for the anhydrous compound and 55°C for the hydrate]. The chloropyruvic acid was stored in a vacuum desiccator over KOH pellets at 0–4°C, when it appeared to be stable for many months.

Chloro[^{14}C]pyruvic acid was made by shaking sodium [^{14}C]pyruvate (2.25 μmol, 25 mCi) (The Radiochemical Centre, Amersham, Bucks., U.K.) with SO_2Cl_2 (0.3 ml) in a tube protected from the air by a $CaCl_2$ tube for 60 h. The solution was then transferred to a vacuum desiccator over KOH pellets for at least 1 week, dry acetone was added and removed, and the residue was dissolved in 0.5 ml of acetone.

This solution was used directly for the inactivation experiments. The molar concentration of [^{14}C]pyruvate compounds (chloropyruvate and any unchanged pyruvate) was determined by the use of lactate dehydrogenase (Barnett *et al.*, 1971) and the specific radioactivity (1.59×10^7 c.p.m./μmol) calculated from this. The proportion of pyruvate was probably very low, but in any case does not affect the specific radioactivity.

Other chemicals and enzymes were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Enzyme assays, protein determinations, chromatography, electrophoresis and inactivation experiments were carried out as previously described (Barnett *et al.*, 1971).

Comparison of the rates of reaction of bromopyruvate and chloropyruvate with N-acetylcysteine. 100mM-Halogenopyruvic acid was adjusted to pH6.0 by NaOH in a pH-stat. The solutions were kept at 9°C and the cell was maintained at this temperature throughout the following operations. *N*-Acetylcysteine (2μmol) was adjusted to pH6.0 in a final volume of 4.9ml. 100mM-Halogenopyruvic acid (0.1ml, 10μmol) was added and the pH maintained at pH6.0 by automatic titration with 0.01M-NaOH. The increase in volume (0.2ml) and the consumption of halogenopyruvate during the reaction were disregarded for the calculation of the rate constants assuming pseudo-first-order kinetics. The rate constants must therefore be regarded as approximate.

Reaction of N-acetylneuraminate lyase with chloro-[^{14}C]pyruvate. Chloro[U-^{14}C]pyruvate (1.2μmol, 1.59×10^7 c.p.m./μmol) in 0.3ml of acetone was incubated with *N*-acetylneuraminate lyase (2.5mg) in 1.0ml of 50mM-potassium phosphate buffer, pH7.2, for 2min at 37°C. The final concentration of chloropyruvate was about 1mM. After this time, the reaction was stopped by addition of 1.0ml of 2mM-dithiothreitol, and the enzyme activity was measured and found to be zero. A control to which the same proportion of acetone was added was fully active. The solution was dialysed overnight, freeze-dried and dissolved in 1ml of water. $NaBH_4$ (8mg) was added to reduce the S-CH_2–CO–CO_2H residue to S-CH_2–CHOH–CO_2H, and the solution was chromatographed on a column (45cm×2.5cm) of Sephadex G-25 with 0.15M-KCl as eluent. The fractions containing the bulk of the radioactivity were combined and the specific radioactivity was measured; it was 2.9×10^5 c.p.m./mg of protein. This corresponds to 4.5 pyruvate residues attached per molecule of enzyme, a molecular weight of 2.5×10^5 being used.

Reduction of the pyruvate–N-acetylneuraminate lyase complex with $NaBH_4$. A sample of the same manufacturer's batch of sodium pyruvate (1.4μmol, 1.37×10^7 c.p.m./μmol) and a sample of the same batch of enzyme (2.5mg) as was used in the previous experiment were incubated in 1.2ml of KCl buffer, pH7.2, for 10min at 25°C. Then 0.1ml of 1M-$NaBH_4$ was added and, after a further 10min, 50μl of acetic acid. The additions of $NaBH_4$ and acetic acid were repeated four times. The enzyme activity was measured and found to be zero. The protein was dialysed overnight at 4°C freeze-dried and applied to the Sephadex G-25 column. The specific radioactivity of the combined fractions containing most of the radioactivity was determined (2.0×10^5 c.p.m./mg of protein). Assuming a molecular weight for the enzyme of 2.5×10^5, this corresponds to 3.7mol of pyruvate/mol of enzyme.

Electrophoresis. Electrophoresis was on Whatman no. 1 paper in a Shandon vertical electrophoresis tank and a Vokam power pack at 300V. The buffers were: 25% formic acid–acetic acid–water (13:29:258, by vol.), pH1.6; pyridine–acetic acid–water (50:2:948, by vol.), pH6.0; sodium diethylbarbiturate–ethylbarbituric acid – water (10.3:1.84:1000, w/w/v), pH8.6. In 100min *S*-3-lactate)-cysteine migrated −3.0cm, +3.0cm and +5.1cm towards the anode respectively in the three different buffers.

Determination of apparent molecular weight. The apparent molecular weights were determined by the method of Andrews (1965).

Results and discussion

Reaction of chloropyruvate and bromopyruvate with N-acetylcysteine and N-acetylneuraminate lyase. As expected, the rate of reaction of chloropyruvate with a simple thiol, *N*-acetylcysteine, was slower than that of bromopyruvate. The approximate second-order rate constants at 9°C were 1.65 and 10.6 litre·mol^{-1}·s^{-1} respectively.

In contrast, chloropyruvate inactivated *N*-acetylneuraminate lyase much more quickly than bromopyruvate (Fig. 1). The difference in rate was tenfold. Inactivation of the enzyme by chloropyruvate appeared to be associated with reaction with a residue at, or close to, the active site because the substrate, pyruvic acid, protected against the inactivation (Fig. 1).

Because of the enhanced rate of reaction with the active site, and the lowered rate of reaction with simple thiols, it seemed that it might be possible to use chloropyruvic acid to react preferentially with groups at the active site of *N*-acetylneuraminate lyase.

Product of the reaction of chloropyruvate with N-acetylneuraminate lyase. After reaction of the enzyme with 1mM-chloro[^{14}C]pyruvate for 2min at 37°C, it was completely inactivated. Under the same conditions bromopyruvate only inactivated the enzyme by about 35% (Barnett *et al.*, 1971). By comparing the specific radioactivity of the inactive protein with that of the chloro[^{14}C]pyruvate, and assuming a molecular weight for the enzyme of 250000, 4.5 pyruvate residues were bound to the enzyme protein. This compared with 3.7 pyruvate residues bound to the

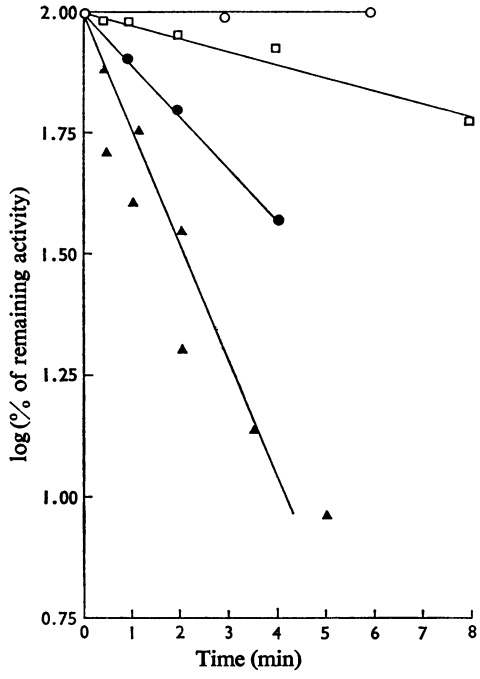

Fig. 1. *Rates of inactivation of N-acetylneuraminate lyase by chloropyruvate and bromopyruvate at 5°C*

The enzyme (10 units) and inactivator were incubated in 0.2ml of sodium phosphate buffer, pH7.2, and, after the times shown, 10μl was pipetted into the standard assay mixture containing 1mM-dithiothreitol at 37°C. The activity remaining was measured. ○, No inactivator; □, 0.5mM-bromopyruvate; ▲, 0.5mM-chloropyruvate (two experiments); ●, 0.5mM-chloropyruvate in the presence of 10mM-pyruvate. 10mM-Pyruvate caused a slight inhibition of the enzyme because of competitive inhibition and this is corrected for in the plot.

same sample of enzyme by $NaBH_4$ reduction of the enzyme–pyruvate Schiff-base complex. The number of pyruvate residues bound by the two methods was therefore very similar, indicating that the chloropyruvate was acting almost exclusively at the residue at or close to the active site. This contrasts markedly with the situation found with bromopyruvate, where 20 residues reacted with each enzyme molecule (Barnett *et al.*, 1971). It would therefore appear that, at least for this enzyme, the specificity of halogenopyruvate inactivation can be markedly increased by using the chlorinated analogue.

Identification of the residue attacked by chloropyruvate. After borohydride reduction to stabilize the pyruvate residue, the product of chloro[^{14}C]pyruvate inactivation was hydrolysed. The sole radioactive product of hydrolysis moved identically with *S*-(3-lactate)-cysteine on electrophoresis at three different pH values. This confirms the previous suggestion (Barnett *et al.*, 1971) that the inactivation of *N*-acetylneuraminate lyase by halogenopyruvate is caused by reaction with a cysteine residue at the active site.

The *S*-CH_2–CO–CO_2H residue formed by the reaction of chloropyruvate with the active-site thiol might still form a Schiff base with the active-site lysine. If this occurred, $NaBH_4$ reduction of the inactivated protein would give a 'bridge' compound between the two amino acid residues. This did not appear to occur to any great extent because no second radioactive spot was detected on electrophoresis of the hydrolysate. It therefore seems probable that covalent attachment of the pyruvate to the sulphur atom of the cysteine forces the pyruvate out of its correct position, so displacing the equilibrium of imine formation.

Molecular weight of N-acetylneuraminate lyase. The apparent molecular weight of the enzyme from *Cl. perfringens* has been determined by the use of Sephadex chromatography (Barnett *et al.*, 1971; DeVries & Binkley, 1972). The two groups of workers obtained values of about 250000 and 92000 respectively. We therefore repeated the determination on the enzyme preparation used in this work, which had been treated more like that of DeVries & Binkley (1972) during preparation, and on an enzyme preparation identical with that used by Barnett *et al.* (1971). The apparent molecular weights were about 100000 and 250000 respectively, indicating that the enzyme can polymerize, and probably exists either as the dimer or tetramer in the two forms. By addition or omission of the DEAE-cellulose step either the dimer or tetramer can be prepared. The electrophoretic results of DeVries & Binkley (1972), which show the presence of two separately migrating forms of active enzyme, have been confirmed in our laboratory (G. Rasool, unpublished work) and support the suggestion that it is a polymeric enzyme.

For the present results on chloropyruvate inactivation, we have used the value of 250000 for the apparent molecular weight, so that the results correlate with those of our previous paper (Barnett *et al.*, 1971). If the value of 100000 is used for the calculation, 1.8 residues of chloropyruvate bind to the enzyme, compared with 1.5 residues of pyruvate bound after reduction of the Schiff-base complex. The departure from the expected value of 2 may be due either to the error in the molecular weight, or to the presence of non-enzymic protein.

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