Inhibition of Glycolytic Enzymes by 2-Phosphotartronate

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2-Phosphotartronate has been synthesized by permanganate oxidation of glycerol 2-phosphate and has been tested as an inhibitor of five glycolytic enzymes that bind phosphoglycerate or phosphoglycollate. Competitive inhibition of rabbit muscle phosphoglycerate mutase, enolase and pyruvate kinase was observed. Triose phosphate isomerase and 3-phosphoglycerate kinase were not inhibited.

Several small chemical analogues of 2-phosphoglycerate of the form $^{-}\text{O}_2\text{C}-\text{CH}(\text{OPO}_3^{2-})-\text{X}$ have been synthesized and tested as inhibitors of the glycolytic enzymes enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40). These analogues (see Table 1) include 2-phosphoglycollate ($\text{X} = \text{H}$), 2-phospholactate ($\text{X} = \text{CH}_3$) and 2-phosphotartronate semialdehyde ($\text{X} = \text{CHO}$). All the compounds are relatively good to excellent analogues of 2-phosphoglycerate and phosphoenolpyruvate, as judged by their low K_i values (relative to K_m). 2-Phosphotartronate ($\text{X} = \text{CO}_2\text{H}$), synthesized by a multi-step method (Brierly, 1961), has been tested as an inhibitor of enolase (Brierly, 1961), alkaline phosphatase (EC 3.1.3.1), glycogen phosphorylase (EC 2.4.1.1) and hexokinase (EC 2.7.1.1) (Walsh, 1963). We now report here a single-step synthesis of 2-phosphotartronate from glycerol 2-phosphate, and the effect of 2-phosphotartronate on several more glycolytic enzymes. A preliminary report of this work has been made (Kokoska & Spring, 1974).

Experimental

The following enzymes were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and were used without further purification: phosphoglycerate mutase; pyruvate kinase, type II; triose phosphate isomerase, type III; glycerol 1-phosphate

dehydrogenase, type X (all from rabbit muscle); and yeast 3-phosphoglycerate kinase, type IV. Rabbit muscle enolase was purified by a modification of the method of Winstead & Wold (1966), with omission of the acetone fractionation step. Enzymes were assayed spectrophotometrically in triplicate at 22°C in a 1 ml assay volume according to previously reported methods: phosphoglycerate mutase (Grisolia, 1962), pyruvate kinase (Pon & Bondar, 1967), triose phosphate isomerase (Norton *et al.*, 1970), 3-phosphoglycerate kinase (Scopes, 1969) and enolase (Winstead & Wold, 1966). The 2,3-diphosphoglycerate phosphatase activity of rabbit muscle phosphoglycerate mutase was assayed by measuring P_i (see below) released in an incubation (15–30 min) of phosphoglycerate mutase (0.3–1.5 units) in 50 mM-Tris/ H_2SO_4 /1 mM- MgSO_4 /0.01 mM-EDTA/1.0 mM-2,3-diphosphoglycerate, pH 7.5 (1 ml). The phosphatase activity was negligible, compared with a non-enzyme control, in the presence or in the absence of 1 mM-phosphoenolpyruvate or 2-phosphotartronate. The activity in the presence of 1 mM-2-phosphoglycollate was 1.46 nmol of P_i released/min per unit of phosphoglycerate mutase activity. K_m and K_i values were calculated from Lineweaver–Burk plots, by using a weighted least-squares analysis to calculate slopes and intercepts.

All substrates were purchased from Sigma Chemical Co., except for DL-2-phosphoglycerate,

Table 1. Enzyme inhibitors of the structure $^{-}\text{O}_2\text{C}-\text{CH}(\text{OPO}_3^{2-})-\text{X}$

These data for rabbit muscle enolase and pyruvate kinase are calculated from inhibition data reported in the references. The $K_{m(\text{app.})}$ values refer to D-2-phosphoglycerate (enolase) and phosphoenolpyruvate (pyruvate kinase).

Compound	X	Enolase $K_i/K_{m(\text{app.})}$	Pyruvate kinase $K_i/K_{m(\text{app.})}$	Reference
2-Phosphoglycollate	H	5.7	2.0	Nowak & Mildvan (1970)
D-2-Phospholactate	CH_3	4.7	0.8	Nowak & Mildvan (1970)
D-2-Phosphotartronate semialdehyde	CHO	0.09	—	Spring & Wold (1971b)
2-Phosphotartronate	CO_2^{-}	6.6	1.5	Present paper

which was synthesized by the method of Kiessling (1935) and converted into the soluble tris(cyclohexylammonium) salt by the method of Winstead & Wold (1966).

2-Phosphotartronate was synthesized by oxidation of glycerol 2-phosphate. Initial attempts to oxidize with bromine at neutral pH yielded little 2-phosphotartronate, even though this method is used successfully for the synthesis of the monocarboxylic acid, 2-phosphoglycerate (Kiessling, 1935). Oxidation was instead performed in alkaline KMnO_4 . A typical procedure began with 13.5 g (50 mmol) of disodium glycerol 2-phosphate pentahydrate in 1 litre of 0.01 M-NaOH, to which was added 63.2 g (400 mmol) of KMnO_4 . The reaction mixture was stirred at room temperature for 10 days (to maximize the yield). The pH of the reaction mixture fell during the reaction, and daily additions of NaOH were made to maintain the pH at 11. The reaction was terminated by the addition of enough methanol to consume the unchanged KMnO_4 , and the MnO_2 precipitate was removed by centrifugation. The supernatant was treated with magnesia solution (Cardini & Leloir, 1957) to remove P_i and then with barium acetate (100 mmol) to precipitate the dibarium 2-phosphotartronate. The barium salt precipitate was collected by centrifugation, washed with water and acetone, and dried *in vacuo*. The barium salt precipitate (13 g, approx. 28 mmol based on a molecular weight of 471 for dibarium 2-phosphotartronate) was resuspended with the sulphonic resin Dowex-50 (H^+ form) in approx. 100 ml of water to remove Ba^{2+} ions. The Dowex-50 was removed by filtration, and the acidic solution was titrated to pH 9 with cyclohexylamine. Acetone (approx. 3 vol.) was added to the turbidity point, and crystallization of the tetra(cyclohexylammonium) 2-phosphotartronate occurred rapidly at -20°C . Additional amounts of pure material could be recovered by adding more acetone to the mother liquor before contamination from 2- and 3-phosphoglycerate occurred. The total yield of pure tetra(cyclohexylammonium) 2-phosphotartronate was 9.5 g (15.9 mmol, or 32% overall yield).

The tetra(cyclohexylammonium) 2-phosphotartronate had m.p. $158\text{--}169^\circ\text{C}$ (decomp.), compared with a previously reported value of $154\text{--}159^\circ\text{C}$ (Brierly, 1961). An elemental analysis of the barium salt for C and H (performed by Chemalytics, Tempe, AZ, U.S.A.) and P (by using H_2SO_4 digestion and the P_i analysis method of Chen *et al.*, 1956) was consistent with the proposed structure (Found: C, 7.9; H, 0.6; P, 7.0; calc. for $\text{Ba}_2\text{C}_3\text{HO}_8\text{P}$: C, 7.7; H, 0.2; P, 6.6%). Paper-chromatographic purity was demonstrated in two solvents and the compound was well separated from possible contaminants; R_F values in an acidic solvent (methanol/acetic acid/water, 16:3:1, by vol.) and a basic solvent [methanol/conc. aq. NH_3 (sp.gr. 0.880)/water, 6:1:3

by vol.] were respectively: 2-phosphotartronate (0.93, 0.62); 2-phosphoglycerate (0.84, 0.71); 3-phosphoglycerate (0.80, 0.66); 2-phosphoglycollate (0.78, 0.64). Spots were detected by the phosphate spray of Hanes & Isherwood (1949). An n.m.r. spectrum was obtained with a Varian T-60 NMR spectrometer (Department of Chemistry, University of Houston) on a 10% (w/v) solution of tetra(cyclohexylammonium) 2-phosphotartronate in $^2\text{H}_2\text{O}$ (99.87%; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Resonances were observed at 4.6δ (singlet), 3.2δ (unresolved, broad single peak) and $1.2\text{--}2.3\delta$ (multiplet) in the proportions 1.0:4.3:44.6. The calculated proportions for the C-2 proton of 2-phosphotartronate (4.6δ), the C-1 protons of cyclohexylamine (3.2δ) and the methylene protons of cyclohexylamine ($1.2\text{--}2.3\delta$) are 1:4:44. The resonances were unaffected in relative area for several hours when the solution was made 0.1 M with respect to NaOH, indicating a lack of base-lability for any of the protons. A water peak at approx. 5δ was also observed in all spectra.

Results and Discussion

Synthesis and characterization of 2-phosphotartronate

The single-step synthetic method chosen here yielded gram quantities of 2-phosphotartronate in pure form, thus simplifying the synthesis over the multi-step method of Brierly (1961). The m.p. (decomp.), elemental analysis, chromatographic analysis and n.m.r. spectrum (see the Experimental section) all confirm the purity and correct identification of the product. Unlike 2-phosphotartronate semialdehyde (Spring & Wold, 1971a), 2-phosphotartronate is quite stable in dilute acids and bases at room temperature, and is unable to exchange its C-2 proton with solvent in 0.1 M-NaOH (see the Experimental section). These properties suggest that 2-phosphotartronate is unlikely to be as potent an enolase inhibitor as 2-phosphotartronate semialdehyde, since the semialdehyde is enolized by chemical or enzymic removal of its C-2 proton and binds to enolase as a tightly held enolate ion (Spring & Wold, 1971b). This prediction is confirmed by the data of Brierly (1961) and by the present work (see below).

Enzyme inhibition studies

Because of its chemical similarity to 2-phosphoglycollate, 2-phosphoglycerate and phosphoenolpyruvate, we have tested 2-phosphotartronate as an inhibitor of several glycolytic enzymes, as indicated in Table 2.

Triose phosphate isomerase is known to be strongly inhibited by 2-phosphoglycollate, because of its resemblance to the *cis*-enediol transition state of the reaction (Wolfenden, 1972). In addition, the enzyme

Table 2. *Competitive enzyme inhibition by phosphotartaric acid*

Enzymes were from rabbit muscle except where noted. Assays were performed spectrophotometrically in triplicate at 22°C by using the assay methods referred to in the Experimental section. The 2-phosphotartaric acid concentrations for inhibition were in the range 0.1–1.0 mM, and 5 mM-Mg²⁺ was present in all assays except that for triose phosphate isomerase to ensure that inhibition was not due to chelation of Mg²⁺ by 2-phosphotartaric acid. K_m , V and K_i values were calculated from a weighted least-squares analysis of $1/v$ and $1/[S]$. All inhibition was competitive. —, No inhibition; N.D., not determined.

Enzyme	Varied substrate	$K_m(\text{app.})$ (mM)	K_i (mM)	$\frac{K_i}{K_m(\text{app.})}$
Triose phosphate isomerase (EC 5.3.1.1)	3-Phosphoglyceraldehyde	N.D.	—	—
3-Phosphoglycerate kinase (yeast) (EC 2.7.2.3)	3-Phosphoglycerate	0.10	—	—
Hexokinase (yeast)* (EC 2.7.1.1)	ATP	0.70	0.58	0.8
Phosphoglycerate mutase (EC 2.7.5.3)	3-Phosphoglycerate	0.77	0.031	0.04
Enolase (EC 4.2.1.11)	2-Phosphoglycerate	0.11	0.73	6.6
Enolase (yeast)† (EC 4.2.1.11)	2-Phosphoglycerate	0.12	5.6	47
Pyruvate kinase (EC 2.7.1.40)	Phosphoenolpyruvate	0.042	0.064	1.5

* Data of Walsh (1963).

† Data of Brierly (1961).

is known to be inhibited by phosphoenolpyruvate (with a K_i/K_m ratio of 7–9:1), but is not affected by 2- or 3-phosphoglycerate (Krietsch *et al.*, 1970); 2-phosphotartaric acid is also without effect (Table 2).

2-Phosphotartaric acid does not compete with 3-phosphoglycerate in the 3-phosphoglycerate kinase reaction, suggesting that the position of the phosphate group is important in binding. On the other hand, Walsh (1963) reported 2-phosphotartaric acid to be an effective inhibitor of hexokinase in competition with ATP. In this case the chemical similarity between ATP and 2-phosphotartaric acid may be the anionic groups, which can bind Mg²⁺, a required cofactor. The inhibition is not due, however, to simple Mg²⁺ chelation (Walsh, 1963). A similar result has been observed for 2,3-diphosphoglycerate, which also inhibits hexokinase (K_i/K_m ratio of 0.3:1) from erythrocytes (Srivastava & Beutler, 1972). This similarity suggests that 2-phosphotartaric acid might serve as a useful analogue of 2,3-diphosphoglycerate in other systems (perhaps haemoglobin or 1,3-diphosphoglycerate mutase).

The unusually strong inhibition of phosphoglycerate mutase by 2-phosphotartaric acid may be due to its similarity to the product of the reaction, 2-phosphoglycerate. Although reliable K_m values for both 2- and 3-phosphoglycerate are hard to obtain for this enzyme, it has been reported (Britton *et al.*, 1972) that the binding of the 2-isomer is tighter than for the 3-isomer. Since the cofactor for the reaction, 2,3-diphosphoglycerate, binds even more tightly than the monophosphoglycerates, the strong inhibition may reflect the structural similarity of 2,3-diphosphoglycerate and 2-phosphotartaric acid. We checked the possibility that the inhibition of activity was due to a stimulation of 2,3-diphosphoglycerate phosphatase activity by 2-phosphotartaric acid. It is known, for example, that 2-phosphoglycolate will stimulate

this phosphatase activity. This would deplete the reaction mixture of needed cofactor and would thus inhibit activity. However, 2-phosphotartaric acid did not stimulate the phosphatase activity over control values (see the Experimental section), thus ruling out this possibility.

The inhibition of enolase by 2-phosphotartaric acid was not unexpected, but the inhibition was much greater for the rabbit muscle than previously reported for the yeast enzyme. This difference may simply be due to differences between the active sites of these two enzymes, as has been observed previously (Wold, 1971).

The inhibition of pyruvate kinase is quantitatively similar to inhibition by other small analogues (Table 1). Several of these analogues have been used as n.m.r. probes of the active site of the enzyme (Nowak & Mildvan, 1972); 2-phosphotartaric acid may also be useful since it contains only one proton.

Although the number of enzymes tested is not exhaustive, the above data suggest that other enzymes that utilize 2-phosphoglycerate, 2,3-diphosphoglycerate, phosphoenolpyruvate or ATP as substrates or effectors can also be inhibited by 2-phosphotartaric acid.

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