Properties of the Phosphonomethyl Isosteres of Two Phosphate Ester Glycolytic Intermediates

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The enzymic synthesis of the 1-phosphonomethyl isostere of fructose 1,6-diphosphate in which the 1-phosphate ($-\text{OPO}_3\text{H}_2$) is replaced by the phosphonomethyl group ($-\text{CH}_2\text{PO}_3\text{H}_2$) is described. The kinetic properties of this fructose diphosphate isostere and of 4-hydroxy-3-oxobutylphosphonic acid, an isostere of dihydroxyacetone phosphate, with aldolase (EC 4.1.2.13), fructose diphosphatase (EC 3.1.3.11) and glycerol phosphate dehydrogenase (EC 1.1.1.8) are described (see Table 1).

Phosphonomethyl isosteres of phosphate ester metabolic intermediates have not been thoroughly investigated (Dixon & Sparkes, 1974). The present paper describes the effects of the substitution of a phosphonomethyl group into fructose diphosphate and dihydroxyacetone phosphate on the maximal velocities and Michaelis constants of aldolase (EC 4.1.2.13), glycerol phosphate dehydrogenase (EC 1.1.1.8) and fructose diphosphatase (EC 3.1.3.11).

It has been demonstrated that, although the substitution of the phosphonomethyl group into dihydroxyacetone phosphate abolishes activity and binding with triose phosphate isomerase (EC 5.3.1.1) (Dixon & Sparkes, 1974), phosphonomethyl isosteres are still bound with high affinity by aldolase, fructose diphosphatase and glycerol phosphate dehydrogenase.†

Materials and Methods

Chemicals were all of analytical grade; enzymes and substrates were obtained from Boehringer (London W5 2TZ, U.K.). 4-Hydroxy-3-oxobutylphosphonic acid (bis-cyclohexylamine salt) was synthesized and kindly given by Dr. H. B. F. Dixon (Dixon & Sparkes, 1974). DEAE-cellulose was purchased as precycled DE-52 from H. Reeve Angel and Co. Ltd. (London E.C.4, U.K.).

Phosphate esters and phosphonates were detected by their capacity to bind Fe^{3+} ions (Wade & Morgan, 1953). Carbohydrates were detected with a AgNO_3 -NaOH dip (Trevelyan *et al.*, 1950).

Rabbit muscle aldolase was assayed at pH 7.5 by coupling the production of dihydroxyacetone phosphate and glyceraldehyde phosphate to the oxidation of NADH by using triose phosphate isomerase and

glycerol phosphate dehydrogenase and following the decrease in absorbance at 340 nm in a Gilford recording spectrophotometer (Stribling & Perham, 1973). Glycerol phosphate dehydrogenase (rabbit muscle) was assayed in a similar system by following the reduction of dihydroxyacetone phosphate by NADH. Rabbit liver fructose diphosphatase was assayed at pH 9.4 by converting the fructose 6-phosphate produced from fructose diphosphate into glucose 6-phosphate and following the reduction of NADP^+ resulting from its oxidation by glucose 6-phosphate dehydrogenase (Pontremoli, 1966).

Absolute concentrations of substrates and isosteres were determined by titration against NAD^+/NADH by using the appropriate enzyme assay system. By this method, over 95% of 4-hydroxy-3-oxobutylphosphonate was acceptable as a substrate for glycerol phosphate dehydrogenase.

Results

Imine formation with aldolase

The class 1 lysine-active-site aldolases form an imine between the ϵ -amino group of lysine and the carbonyl group of dihydroxyacetone phosphate in the catalysis of the aldol condensation reaction (for review see Lai & Horecker, 1973). This imine may be reduced by borohydride, which irreversibly blocks the active site with a concomitant inhibition of aldolase. The effect of NaBH_4 on rabbit muscle aldolase in the presence of the phosphonomethyl isostere of dihydroxyacetone phosphate was determined as follows.

Rabbit muscle aldolase (1 mg) and 1 μmol of either dihydroxyacetone phosphate or its phosphonomethyl isostere in 1 ml of 10 mM-Tris-HCl-1 mM EDTA, pH 7.5, were incubated at 0°C for 10 min before the addition of 2 μl of octanol (to minimize foaming), 0.25 ml of 0.5 M-2-(*N*-morpholino)ethanesulphonic acid (adjusted to pH 5.85 with solid Tris)

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† See note added in proof.

and 0.25 ml of 0.25 M-NaBH₄. The mixture was allowed to incubate at 0°C for a further 10 min before the reaction was stopped by the addition of 0.25 ml of 0.4 M-Tris-HCl, pH 9.0. The reagents were removed by dialysis overnight against 10 mM-Tris-HCl, pH 7.5. The treated enzyme was assayed and the specific activity/mg of protein added was determined. Enzyme that had been treated with NaBH₄ without either dihydroxyacetone phosphate or its isostere retained over 95% of its original activity, but treatment with NaBH₄ plus dihydroxyacetone phosphate or NaBH₄ plus isostere diminished the activity to 15 and 18% respectively. The residual activity in each case was equally sensitive to further treatment with borohydride and substrate. It appears from this experiment that the isostere of dihydroxyacetone phosphate, 4-hydroxy-3-oxobutylphosphonate, is bound in the customary active-site complex by rabbit muscle aldolase. The only other compound known to mimic the natural substrate in this manner is hydroxyacetone phosphate (Rose & O'Connell, 1969).

Synthesis of the 1-phosphonomethyl isostere of fructose 1,6-diphosphate

The 1-phosphonomethyl isostere of fructose 1,6-diphosphate was synthesized from 4-hydroxy-3-oxobutylphosphonate by using rabbit muscle aldolase to catalyse the condensation with glyceraldehyde phosphate.

D-Glyceraldehyde 3-phosphate (2 μ mol) and 0.1 mg of aldolase were incubated in 1 ml of 10 mM-Tris-HCl, pH 7.5, at 20°C with 4 μ mol of either dihydroxyacetone phosphate or its isostere. After 20 min samples were subjected to electrophoresis (5 kV or about 100 V $\cdot$ cm⁻¹, for 20 min) in pyridine (1%, v/v)-acetic acid (10%, v/v), pH 3.5, on Whatman 3MM paper cooled in white spirit. The papers were dried and tested for carbohydrates and phosphates. When dihydroxyacetone phosphate was used, a phosphorylated carbohydrate with the mobility of fructose 1,6-diphosphate ($M_{Fm-1.6-P_2} = 1.0$) was formed, together with a trace of material corresponding to fructose monophosphate ($M_{Fm-1.6-P_2} = 0.7$). This was probably fructose 1-phosphate, formed by the condensation of dihydroxyacetone phosphate with some glyceraldehyde released by the hydrolysis of glyceraldehyde 3-phosphate. With the analogue, a compound of slightly slower mobility than fructose diphosphate ($M_{Fm-1.6-P_2} = 0.95$) was formed, but it did not appear in the control without aldolase.

On a preparative scale, D-glyceraldehyde 3-phosphate (100 μ mol) was incubated with 4-hydroxy-3-oxobutylphosphonate (150 μ mol) and 10 mg of rabbit muscle aldolase in 10 ml of 10 mM-Tris-HCl, pH 7.5, for 1 h at 20°C. The reaction was terminated by the addition of 1 ml of 10% (v/v) trichloroacetic acid and the precipitated protein removed by centrifugation.

The supernatant was adjusted to pH 4.6 with a dilute solution of pyridine and applied to a column (30 cm $\times$ 2 cm) of DEAE-cellulose equilibrated with 0.18 M-pyridine-0.36 M-acetic acid, pH 4.6. The column was eluted with the same buffer and fractions (10 ml) were collected. The fractions were spot-tested for carbohydrate and samples of positive fractions electrophoresed at pH 3.5, 5 kV, for 20 min with fructose diphosphate as a marker to identify the product. Two peaks were eluted from the column: the first, at approx. 1½ column volumes, contained monophosphates; the second, at 4 column volumes, contained the putative isostere of fructose diphosphate. The second peak was pooled (30 ml) and concentrated by rotary evaporation. The bath temperature was restricted to 35°C to avoid oxidation of the product. The pyridine-acetate buffer was removed by repeated addition of more water until a solution of the pyridine salt of the phosphonomethyl isostere remained. The product was converted into the free acid form by applying the solution to a small column (1 cm $\times$ 5 cm) of the sulphonated polystyrene resin Dowex 50 (H⁺ form).

After it had been ascertained that 4-hydroxy-3-oxobutylphosphonate could be titrated against NADH by using glycerol phosphate dehydrogenase, the putative isostere was titrated in the aldolase assay to demonstrate an overall yield of 85% from the aldol condensation with D-glyceraldehyde phosphate. The product, which gave a single spot on electrophoresis ($M_{Fm-1.6-P_2} = 0.95$), contained no 4-hydroxy-3-oxobutylphosphonate or glyceraldehyde phosphate, since there was no consumption of NADH in the titration in the absence of aldolase.

A sample of the isostere of fructose diphosphate was treated with alkaline phosphatase at pH 8.0. The product, on electrophoresis, corresponded in mobility to that predicted for the 1-phosphonomethyl isostere of fructose 1-phosphate ($M_{Fm-1.6-P_2} = 0.65$).

Investigation of the enzymic properties of the phosphonomethyl isosteres

(a) *Kinetics of glycerol phosphate dehydrogenase.* The initial rates of reaction with a range of concentrations of either dihydroxyacetone phosphate or its isostere in a total volume of 2.5 ml of 50 mM-Tris-HCl-1 mM-EDTA, pH 7.5, containing 6 μ mol of NADH and 5–20 μ g of glycerol phosphate dehydrogenase, were determined. The reactions were started by the addition of substrate with simultaneous stirring of cuvette contents. The maximum velocities and Michaelis constants were determined in triplicate from Lineweaver-Burk plots and the data are summarized in Table 1. Both substrates showed a slight inhibition of the enzyme at high concentrations; this effect has previously been described for the natural substrate (Borrebaek *et al.*, 1965). Adams *et al.*

Table 1. Comparison of the kinetic constants of substrates and their isosteres

For details see the text.					
Enzyme	Substrate	K_m for substrate	K_m for isostere	$\frac{K_m \text{ for isostere}}{K_m \text{ for substrate}}$	$\frac{V \text{ for isostere}}{V \text{ for substrate}}$
Glycerol phosphate dehydrogenase	Dihydroxyacetone phosphate	$0.4 \pm 0.05 \text{ mM}$	$0.4 \pm 0.05 \text{ mM}$	1	0.1 ± 0.01
Fructose diphosphatase	Fructose diphosphate	$4 \pm 0.5 \mu\text{M}$	$70 \pm 5 \mu\text{M}^*$	17.5*	less than $\delta^\dagger$
Aldolase	Fructose diphosphate	$2 \pm 0.2 \mu\text{M}$	$2 \pm 0.2 \mu\text{M}$	1	0.05 ± 0.005

* The value entered here is the K_i of the isostere in inhibiting hydrolysis of the substrate.

$^\dagger \delta$ is the lower limit of measurement.

Fig. 1. Determination of K_i for the 1-phosphonomethyl isostere of fructose 1,6-diphosphate with fructose diphosphatase

The initial rates of reaction of fructose diphosphatase with a series of concentrations of fructose diphosphate and its isostere were determined. Fructose 1,6-diphosphate concentrations used were: $\circ$, $90 \mu\text{M}$; Δ , $36 \mu\text{M}$; $\square$, $18 \mu\text{M}$; $\bullet$, $9 \mu\text{M}$. K_i was determined from the intersection of the plots by the method of Dixon (1953).

(1974) have independently studied the reverse reaction with this enzyme and the isostere of D-glycerol 3-phosphate.

(b) *Kinetics of rabbit muscle aldolase with fructose 1,6-diphosphate and its 1-phosphonomethyl isostere.* The 1-phosphonomethyl isostere is cleaved by aldolase to 4-hydroxy-3-oxobutylphosphonate and glyceraldehyde 3-phosphate. Triose phosphate isomerase, which is added to the normal aldolase assay to interconvert the two triose phosphates, was omitted to avoid the problems that could arise from glycerol phosphate dehydrogenase being presented with a mixture of dihydroxyacetone phosphate and its isostere. The amount of glycerol phosphate dehydrogenase in the assay was increased to $100 \mu\text{g}$ per assay to compensate for the lower activity of the enzyme with the isostere. These modifications produced an assay that depended solely on aldolase activity over

the whole range of substrate concentrations used. The K_m and V values determined from Lineweaver-Burk plots are summarized in Table 1.

(c) *Properties of the 1-phosphonomethyl isostere of fructose diphosphate as an inhibitor of fructose diphosphatase.* The 1-phosphonomethyl isostere of fructose diphosphate has a stable C-C-P bond in place of the customary phosphate ester link. The effect of this substitution on the affinity of the enzyme for fructose diphosphate was evaluated by determining K_i for the isostere. The initial rates of reaction of fructose diphosphatase ($20 \mu\text{g}$) with fructose diphosphate (0 – $40 \mu\text{M}$) in the presence of the isostere (0 – 0.5 mM) were measured at pH 9.4. The isostere proved to be a competitive inhibitor (Fig. 1), with K_i $70 \mu\text{M}$.

Discussion

The kinetic data summarized in Table 1 illustrate that the substitution of a phosphonomethyl group in dihydroxyacetone phosphate and in the 1-position of fructose 1,6-diphosphate does not seriously affect their binding to the enzymes aldolase and glycerol phosphate dehydrogenase, neither of which involves the hydrolysis of the modified group. A similar pattern was observed with the phosphonomethyl isostere of D-glycerol 3-phosphate as a substrate for glycerol phosphate dehydrogenase (Adams *et al.*, 1974). It is therefore somewhat surprising that the maximal velocities of the enzymes with the isosteres are diminished by about an order of magnitude with respect to their natural substrates. This may suggest that, although the K_m values of the phosphonomethyl isosteres are not affected by the substitution, the decrease in maximal velocity is due either to a very slight change in the phosphate group, which has an essential role in catalysis, or to differences in the enzyme conformation induced by the binding of the isostere as opposed to the natural substrate.

Following the suggestion (Orr & Knowles, 1974) that one major difference between phosphonates and phosphates derives from the higher second pK value,

the effect of varying the pH on the V values of glycerol phosphate dehydrogenase and aldolase with the natural substrates and with the isosteres was investigated. There was no significant difference in the ratio of maximal velocities at pH 7.2 and pH 8.2 with isosteres and phosphates when the activities of the two enzymes were compared.

The substitution of the 1-phosphonomethyl group into fructose diphosphate abolishes all measurable activity with fructose diphosphatase, which hydrolyses the 1-phosphate of the natural substrate. The affinity of the enzyme for the isostere is decreased by about an order of magnitude, if in this case K_m provides an upper limit for K_s (Dalziel, 1962).

These three observations contrast sharply with the properties of triose phosphate isomerase, which shows no activity with or affinity for 4-hydroxy-3-oxobutylphosphonate (Dixon & Sparkes, 1974).

The synthesis of the 1-phosphonomethyl isostere of fructose diphosphate may provide a useful addition to the range of inhibitors available for fructose diphosphatase since, unlike the majority, it is not permanently fixed in one anomeric configuration.

Note Added in Proof (Received 21 May 1974)

Since the submission of this paper, similar data have been published (Cheng *et al.*, 1974) on the activity of

glycerol phosphate dehydrogenase with 4-hydroxy-3-oxobutylphosphonate.

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